

STUDIES ON IMMUNITY TO PASTEURELLA HAEMOLYTICA

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List of Abbreviations

$^3\text{H}$	=	tritiated thymidine
%	=	percentage
xg	=	relative centrifugal force
%K	=	percentage killing
$\alpha$	=	alpha
$\beta$	=	beta
$\gamma$	=	gamma
$\mu\text{Ci}$	=	micro Curie
$\mu\text{l}$	=	microliter
$\mu\text{g}$	=	microgram
$\mu\text{M}$	=	micron
pg	=	picogram
BALT	=	bronchus associated lymphoid tissue
BAM	=	bronchoalveolar macrophage
$^{\text{C}}_{\text{O}}$	=	carbon
$^{\circ}\text{C}$	=	degrees centigrade
ccu	=	colour changing units
cfu	=	colony forming units
CIE	=	crossed immunoelectrophoresis
CMI	=	cell mediated immunity
CN	=	cytotoxin neutralisation/cytotoxin neutralising
CPM	=	counts per minute
EDTA	=	ethylene diamine tetra acetic acid
ELISA	=	enzyme-linked immunosorbent assay
Eu	=	endotoxin units
FBS	=	fetal bovine serum
GALT	=	gut associated lymphoid tissue
$\text{H}_2\text{O}_2$	=	hydrogen peroxide
HKO	=	heat killed organisms
HRP	=	horse radish peroxidase
IgA	=	immunoglobulin A
IgD	=	immunoglobulin D
IgE	=	immunoglobulin E
IgG	=	immunoglobulin G
IgM	=	immunoglobulin M
IHA	=	indirect haemagglutination assay
in	=	intranasal
i.p.	=	intraperitoneal
IRP	=	iron restricted protein
kDa	=	kilodaltons
KDO	=	3-ketodeoxyoctonic acid
l	=	litre
LPS	=	lipopolysaccharide
LT	=	heat-killed toxin
M	=	molar
mA	=	milliamps
MBB	=	modified barbitol buffer
mg	=	milligram
min	=	minute(s)

ml	=	milliliter
mM	=	millimolar
MO	=	monocyte
n	=	number of items in a sample
NA	=	not available
NaCl	=	sodium chloride
NB	=	nutrient broth
OD	=	optical density
OMP	=	outer membrane protein
OpI	=	opsonic index
p	=	limit of statistical significance
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate buffered saline
PI3	=	parainfluenza type 3 virus
PMNL	=	polymorphonuclear leucocyte
PWE	=	phenol water extract
Rf	=	relative mobility to the dye front
RPMI	=	Rosewell Park Memorial Institute
s.c.	=	subcutaneous
SDS	=	sodium dodecyl sulphate
S.E.	=	standard error of the mean
sIgA	=	secretory immunoglobulin A
SPF	=	specific pathogen free
SSE	=	sodium salicylate extract
ST	=	heat-stable toxin
TCID <sub>50</sub>	=	fifty percent tissue culture infective dose
TRIS <sub>50</sub>	=	(Tris (hydroxymethyl) aminomethane)
U.K.	=	United Kingdom
USA	=	United States of America
V	=	volts
w/v	=	weight of solute in volume of solution

## SECTION 1.

General Introduction.

## 1.1. The importance of ovine pneumonic pasteurellosis.

In recent years the sheep sector of the U.K. farming industry has continued to expand (Table 1.1). Amongst the infectious diseases affecting livestock, respiratory diseases are of major importance. They cause considerable economic loss with 8 percent (%) of all sheep deaths in 1982 being due to respiratory disease (ADAS 1982). Losses from respiratory disease, are however, due not only to deaths, but also to debilitation through chronic respiratory disease, generally recognised as unthriftiness. Further evidence for the importance of pneumonia has been gained from slaughter-house surveys in which 5 to 80% (an average of 20%) of lungs were found to be pneumonic on any one day (Sharp, 1977).

Pneumonic pasteurellosis, caused by the Pasteurella species P. haemolytica or P. multocida, has been identified as the most common form of fatal respiratory disease in sheep accounting for 70% of diagnosed pneumonias (MAFF.1980).

P. haemolytica is the most common Pasteurella species isolated from cases of ovine pasteurellosis in Britain (Gilmour, 1980), and is also a frequent isolate from cattle in Britain (Quirie et al., 1986) and in northern America (Carter, 1973; McKercher, 1978) where it is associated with a form of bovine pasteurellosis termed shipping fever complex (Lillie, 1974). Ovine pasteurellosis has also been reported in many other parts of the world including East Africa (Pegram et al., 1979) New Zealand (Salisbury, 1957) Norway (Mohn and Utkler, 1974) and South Africa (Cameron, 1966).

Table 1.1 Sheep and lambs total (U.K.)<sup>a</sup>

Year	1982	1983	1984	1985
numbers	33,067,192	34,069,367	34,801,982	35,627,653

<sup>a</sup> reprinted from Agricultural Statistics. 1986. MAFF, DAFFS, DANI, Welsh Office. Published by HMSO.



With the continued expansion of the sheep industry and further livestock intensification the incidence of disease in general and pasteurellosis in particular might be expected to rise. Antibiotic treatment as a therapeutic measure against pasteurellosis is impractical as it requires excessive livestock handling and is prohibitively expensive to sheep farmers. As both a prophylactic and a therapeutic measure antibiotic treatment also poses an undesirable risk in the selection of antibiotic-resistant mutants. This problem has become apparent in the U.S.A., where inappropriate use of antibiotics by cattle farmers has produced *P. haemolytica* strains carrying plasmid-borne resistance factors (Zimmerman and Hirsh, 1980).

The most appropriate means of controlling ovine pasteurellosis would therefore seem to be by vaccination. However, despite the widespread use of the currently available vaccines doubts about their efficacy have been expressed (Gilmour, 1980; Gilmour *et al.*, 1983) and deaths of both ewes and lambs in vaccinated flocks have been reported (ADAS, 1984). This has prompted further research to improve and develop new vaccines against ovine pasteurellosis and this thesis forms part of the work of this project.

A review of the current knowledge available on the antigens of *P. haemolytica*, the experimental models of pasteurellosis, the mechanisms of immunity by which the mammalian host responds to vaccination and infection and their modulation by *P. haemolytica* was considered to be helpful in determining the research to be undertaken in this thesis.

1.2. P. haemolytica: a Gram-negative organism.

a) Classification of the Genus Pasteurella.

Bacteria in the genus Pasteurella are Gram-negative (Gracilicutes), facultatively anaerobic rods belonging to the family Pasteurellaceae (Bergey, 1984).

P. haemolytica is a cocco-bacillus which is haemolytic on sheep-blood agar, grows on MacConkey agar and does not produce indole. These characteristics differentiate it from P. multocida.

Isolates of P. haemolytica can be divided into two biotypes (A and T) on the basis of morphological and biochemical characteristics (Smith, 1961). Biotype A strains ferment arabinose but usually not trehalose, whereas all biotype T strains ferment trehalose. On agar plate cultures, biotype A colonies are an even grey colour while those of the biotype T are generally larger and more mucoid, with a brownish centre. Taxonomic studies by Smith and Thal (1965) and investigations of nucleic acid homology by Biberstein and Francis (1968) demonstrated marked differences between A and T biotypes which led them to suggest that these biotypes are, in fact, different species.

Within the A and T biotypes 15 different serotypes (Fraser et al., 1982a) have been identified by an indirect haemagglutination (IHA) test (Biberstein et al., 1960), the specificity of which is now known to reside within the capsular antigens (Adlam et al., 1984). Serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13 and 14 belong to the A biotype while serotypes 3, 4, 10 and 15 are T biotype. Additionally, there are isolates that are untypable by the IHA test and so far these have all been of the A biotype (Donachie et al., 1984a).

The A and T biotypes are associated with different types of ovine pasteurellosis. The A biotype principally causes an enzootic pneumonia in all ages of sheep, although lambs under two months of age may develop a septicaemic form of disease. The T biotypes cause a distinct form of systemic disease which is a cause of death in older sheep and was first described by Stamp *et al.* in 1955.

In surveys, serotype A2 has been found to be the most common isolate from cases of ovine pasteurellosis (Thompson *et al.*, 1977; Fraser *et al.*, 1982b). In contrast, the serotype A1 is the predominant isolate in bovine pneumonic pasteurellosis (Wessman and Hilker, 1968; Fox *et al.*, 1971; Mwangota *et al.*, 1978; Allan *et al.*, 1985). Differences in the prevalence of serotypes in disease has led to suggestions that variations in virulence may occur between serotypes, but Gilmour *et al.*, (1986) failed to show any clear differences in pathogenicity between serotypes A1, A2, A7 and A9 in sheep.

b) The immunochemistry of the cell surface and extracellular antigens of Gram-negative bacteria.

The Gram-negative cell-wall consists of a distinct outer-membrane which lies external to a thin layer of peptidoglycan. Together with the underlying cytoplasmic membrane these layers form the Gram-negative cell envelope. The structure of this envelope (Fig.1.1) has been described in most detail for the *Enterobacteriaceae* and has been reviewed by Costerton, (1974) and Rogers *et al.*, (1981).

The cytoplasmic membrane: this trilaminar membrane encloses the cytoplasmic contents. Its structure is similar to that of other biological membranes (Martin and MacLeod, 1971) in that it consists

of a phospholipid bilayer amongst which is dispersed intramembrane and transmembrane proteins. The bacterial cytoplasmic membrane is the site of synthesis and transport of cell wall materials which are assembled outwith the cytoplasmic membrane and it also bears receptor proteins involved in protein uptake (Costerton et al., 1974).

The peptidoglycan layer: this lies exterior to the cytoplasmic membrane and is an inelastic surround which is important in maintaining the shape of the bacteria and withstanding the osmotic pressures of the cytoplasm which might otherwise cause lysis.

The peptidoglycan (Fig 1.1) is a thin layer in the Gram-negative bacterial wall when compared to that found in the Gram-positive wall. Peptidoglycan consists basically of three parts 1) a glycan backbone, composed of a  $\beta$  1-4-linked disaccharide repeating unit of N-acetylglucosamine and  $\beta$  1-4 N-acetylmuramic acid; 2) a linear tetrapeptide which usually includes diaminopimelic acid. The tetrapeptide is attached to the lactyl group of the muramic acid. 3) there is also a peptide cross-linkage which connects adjacent tetrapeptide chains. Together these linkages form a single basket-like molecule of peptidoglycan which encompasses the whole cell. Each of the regions is subject to compositional variations which lead to antigenic diversity, but the nature of the cross-linkage is the most variable feature of the structure (Schleifer and Stackebrandt, 1983). The peptidoglycan layer is attached via a low molecular mass lipoprotein (7.2 kDa in Escherichia coli) to a trilaminar outer membrane (Braun, 1973).

The outer-membrane: this consists of a bilayer which was first discovered by electron microscopy (Birch-Anderson et al., 1953;

Murray, 1957). A number of surface-labelling studies have shown that the internal leaf of the outer membrane is phospholipid in nature whilst the external leaf appears to consist largely of lipopolysaccharide (LPS) (reviewed by Nikaido and Vaara, 1985). A general structure for LPS, proposed by Luderitz *et al.*, (1971), shows it as a branching "O" antigen-specific side chain (somatic antigen), a core region and a lipid A portion (Fig 1.2). The O-antigen, because of the variety of structural forms, has serological specificity and rough and smooth type LPS mutants have been described for many Gram-negative species. The smooth type bears long, branching O-antigen chains and the rough-type mutants lack the branching O-antigen. The core region contains sugars unique to prokaryotes, in particular an aldoheptose and a 3-keto deoxyoctonic acid (KDO), the latter linking the core region to lipid A. The lipid A portion is hydrophobic and thought to be embedded in the inner area of the outer leaflet of the trilaminar outer membrane, whilst the O-antigen polysaccharide region is hydrophilic and is presented on the outer surface of the outer membrane (Fig 1.1).

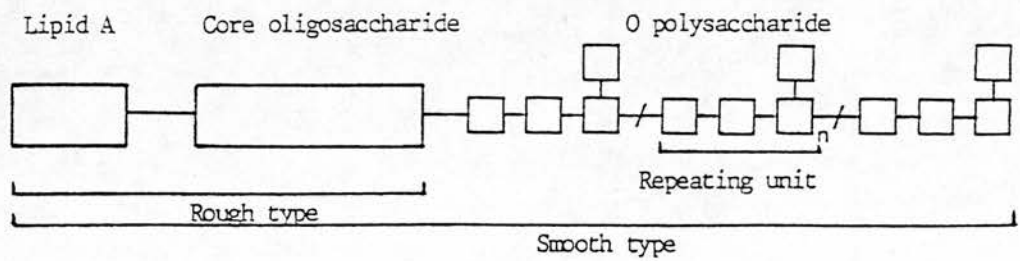
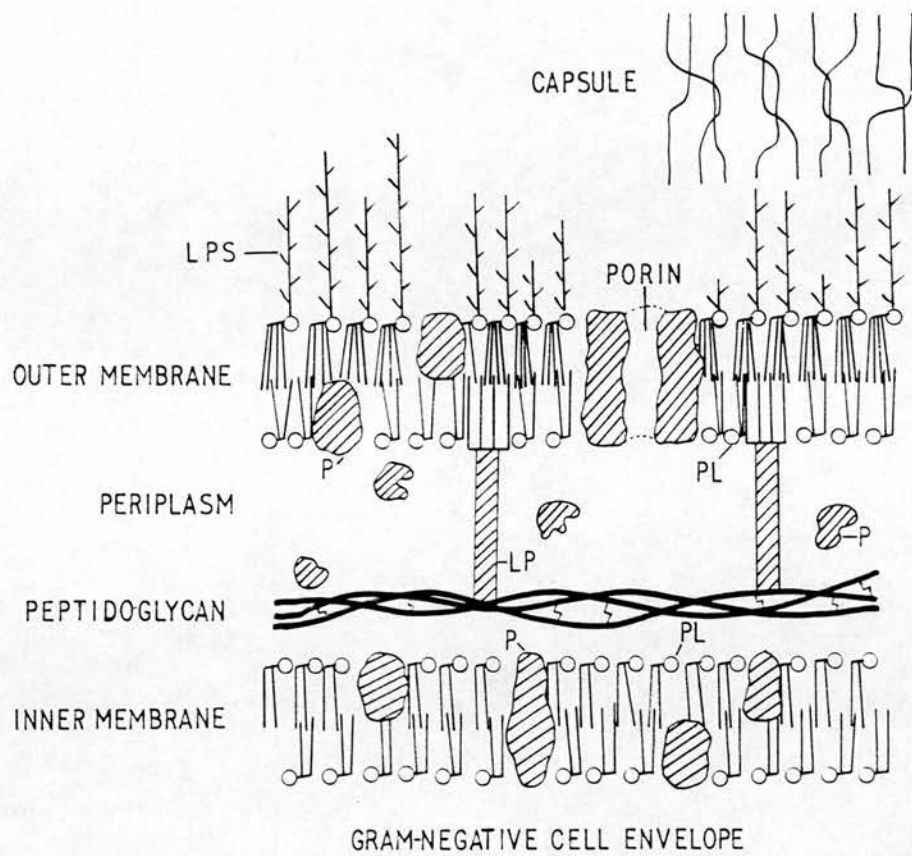
Fifty percent of the outer membrane consists of proteins, some of which (the porins) control the diffusion of solutes across the membrane. The outer membrane, however, is not a site of active transport but rather acts as a molecular sieve (Costerton, 1979). Some outer membrane proteins (OMP) such as the OMP A protein of *E.coli* are external phage receptors (Palva, 1979). while others such as the iron restricted proteins (IRP) are involved in iron scavenging mechanisms and are inducible under certain conditions such as those occurring *in vivo* (Hancock *et al.*, 1976).

Fig. 1.1. A schematic diagram of the Gram-negative cell envelope.

LPS = Lipopolysaccharide, P = Protein,  
LP = Lipoprotein, PL = Phospholipid

Fig. 1.2. A schematic diagram of the lipopolysaccharide molecule. Poxton and Arbuthnott, 1990.







Both the LPS and OMPs of the outer membrane are potential antigens and virulence factors of immunological and pathological importance. The lipid A region of LPS is particularly accredited with causing many pathophysiological effects (Majde and Person, 1981) and LPS was termed endotoxin to distinguish it from the exotoxin of Vibrio cholera by Pfeiffer in 1892. However, the physical state, namely the degree of aggregation of purified LPS, has a profound effect on its biological activity (Luderitz et al., 1982), and this may compromise toxicity studies undertaken with the purified molecule.

Bacterial glycocalyx: external to the envelope components, many Gram-negative bacteria possess exopolysaccharide capsular layers, which are included under the blanket term of a glycocalyx (Costerton et al., 1981). Capsules can be visualised by light microscopy, by negative staining with indian ink (Duguid, 1951) or the congo red-acid fuchsin method (Maneval, 1941). Capsules can also be stained with ruthenium red (Howard and Gourley, 1974) or cationic ferritin (Weiss et al., 1979) for examination by electron microscopy. Exopolysaccharides can be divided into homo- and hetero- polysaccharides. Homopolysaccharides are composed of single-sugar, long-chain molecules such as cellulose, levan and glucan polymers. The sialic acid (N-acetylneuraminic acid) based polymer found in the E. coli K1 (McGuire and Binkley, 1964) and Neisseria meningitidis group B (Bhattacharjee et al., 1975) capsules is also a homopolysaccharide.

Heteropolysaccharides such as those found in Klebsiella species are composed of two or more monosaccharides and frequently contain uronic acids. P. multocida group A has a hyaluronic acid based

capsule which is a linear disaccharide of D-glucuronic acid-  $\beta$  (1-3) N-acetyl-D-glucosamine and which may be involved in avoidance of phagocytosis (Maheswaran and Thies, 1979).

Capsular antigens serve as important serotyping antigens for some Gram-negative organisms such as Shigella, Pasteurella, Neisseria, Klebsiella species and E. coli and may play an important role in pathogenicity.

Other factors such as fimbriae, sex pili and flagella protrude from the the cell wall and play important roles in attachment, plasmid transfer and motility respectively. Flagella are open protein-lattice structures specialised at the proximal end into a hook and inserted into specialised sites in the cell-wall and cytoplasmic membrane (McGroarty et al., 1973). Fimbriae and pili are used synonymously to describe those filamentous appendages which insert into the cell-wall (Ottow, 1975). A distinction based on function has however since been proposed by Jones and Isaacson (1983) in which fimbriae are defined as those appendages involved in bacterial adhesion and colonisation and pili are filaments which are involved in gene transfer.

Flagella are used in serogrouping of some bacterial species such as Salmonella species and E. coli. They are also associated with colonisation and pathogenicity allowing some pathogens such as V. cholerae to respond chemotactically to nutrient gradients (Freter et al., 1981) and others such as Bordetella avium to progress in the opposite direction to mucocilliary clearance (Arp and Cheville, 1984).

The presence of fimbrial types has been closely associated with host specific pathogenicity of enterotoxigenic E. coli strains.

Fimbriae bearing the K88, K99 or colonisation factor antigens (CFA) are associated with adherence and proliferation (colonisation) in the intestine of pigs, cattle and man respectively (Gaastra and de Graaf, 1982).

Extracellular bacterial products: Gram-negative bacteria release many extracellular products into their external environment. These products include exotoxins, endotoxin, proteases and enzymes, many of which are virulence factors and can be used to specifically identify virulent strains of bacteria. In contrast to endotoxin which is a heat-stable LPS, exotoxins are usually heat-labile proteins. Protein toxins such as the *E. coli* heat-labile and heat-stable enterotoxins (LT and ST), cholera toxin, pseudomonas exotoxin A, shiga toxin and pertussis toxin are sub-unit toxins which consist of two types of protein chain. *E. coli* LT and ST have been found to be encoded by plasmids. However, more recently an LT-II toxin which is encoded by chromosomal genes has been identified (Green *et al.*, 1983).

*E. coli* LT and cholera toxin are antigenically similar and may have evolved by plasmid transfer. They activate intracellular adenylate cyclase, which promotes water efflux from gut epithelial cells causing diarrhoea. Pseudomonas exotoxin A and shiga toxin also act intracellularly by inhibiting protein synthesis (reviewed by Eidels *et al.*, 1983 and Middlebrook and Dorland, 1984).

Enzymes released by Gram-negative organisms include proteases. *N. meningitidis*, *N. gonorrhoea* and *E. coli* produce proteases that specifically split IgA (Plaut *et al.*, 1975) which may help these organisms to evade mucosal immunity. Most proteases such as those of *Pseudomonas aeruginosa* are, however, probably involved in

aiding the bacterium to obtain nitrogenous nutrients (Moriyama, 1964).

Haemolytic substances can be divided into heat-labile proteins including phosphorylases and the heat-stable haemolytic lipid or lipoid substances. *P. aeruginosa*, for instance, produces two haemolysins, one a protein haemolysin which is a phospholipase C (Esselmann and Liu, 1961), whilst the other is a glycolipid composed of two molecules of each of rhamnose and  $\beta$ -hydroxydecanoic acid (Jarvis and Johnston, 1949).

With the discovery of many exotoxins produced by Gram-negative bacteria it was no longer possible to consider that endotoxin alone was responsible for pathogenicity, but rather that a number of cellular and extracellular products were involved.

c) The immunochemistry of the cell-surface and extracellular antigens of *P. haemolytica*.

A phenol/water extract (PWE) (Westphal, 1952) of *P. haemolytica* serotypes A1, A2 and A6 cell-wall was prepared by Donachie et al. (1984b) and found to contain both KDO and heptoses, indicating the presence of LPS. A sugar analysis by gas chromatography identified the presence of mannose, galactose, glucose, two unusual heptose sugars, glucosamine and galactosamine. Gel-filtration chromatography of partially acid-hydrolysed A1 PWE suggested that the LPS was of the rough type, consisting of only a short O-antigen. The PWE was also found to contain a serotype-specific antigen which was detectable by the IHA test. However, on crossed immunoelectrophoresis (CIE), two precipitating antigens were identified, of which, only one was serotype specific. Adlam et al. (1985a) have since investigated highly purified capsular extracts

of P. haemolytica and concluded that serotype specificity resides within this capsular antigen.

Perry and Babiuk (1984) investigated the composition of the P. haemolytica serotype T4 LPS and concluded that it is a smooth type LPS with an unbranched, linear O-polysaccharide repeating unit of D-galactose. The core consists of rhamnose, galactose, glucose, two forms of glycerol-D-mannoheptose, KDO and phosphate, while the lipid A fraction contains myristic and  $\beta$ -hydroxymyristic acids, 2-amino-2-deoxy-D-glucose and phosphate. The structure of the LPS from the A and T serotypes so far investigated therefore suggests that the LPSs of the A biotype serotypes are of a rough type while those of the T biotype serotypes may be smooth.

P. haemolytica LPS has been demonstrated to have dermonecrotic effects, haemodynamic effects, including the induction of a biphasic leucocyte response (Keiss, 1964), cause lung lesions in sheep similar to those seen in cases of pneumonia (Brogden et al., 1984) and produce marked changes in carbohydrate metabolism of sheep (Bruss et al., 1983).

The capsular polysaccharide of P. haemolytica A1 has a structure similar to that of the enterobacterial common antigen (Adlam et al., 1984) and is a polymer of N-acetyl-D-mannosaminuronic acid linked 1,4 to N-acetyl-D-glucosamine. The capsules of the T4 and T15 serotypes (Adlam et al., 1985a and Adlam et al., 1985b respectively) have also been identified and are both teichoic acids. The T15 capsule is identical to the K62 capsular polysaccharide of E. coli (Jann and Schmidt, 1980) and the N. meningitidis group H polymer (Van der Kaaden et al., 1984). The T4 capsule differs only in that a phosphate is attached to the C4 of



galactose instead of the C6 as in the T15 polymer. This difference is, however, sufficient to give these capsules different antigenic specificities.

Neither flagella nor fimbriae have yet been identified in P. haemolytica and the role of attachment in the pathogenicity of this respiratory pathogen is uncertain.

Taxonomic studies by Smith and Thal (1965) and investigations of nucleic acid homology (Biberstein and Francis, 1968) revealed marked differences between the A and T biotypes of P. haemolytica and led the latter authors to suggest that the biotypes were different species. Sutherland and Donachie (1985) however showed that ovine isolates of both biotypes produce a cytotoxin which is peculiar to P. haemolytica and is therefore a shared characteristic which supports the continued classification of these two biotypes within the one species. The cytotoxin has not yet been purified and its composition is therefore undetermined. It is, however, known to be a specific leucotoxin and its activity can be abrogated by treatment with trypsin (Baluyut et al., 1981; Sutherland and Redmond, 1986), periodate and amylase (Chang et al., 1986), suggesting that the cytotoxin has both protein and carbohydrate moieties. Since this cytotoxin is leucotoxic it has a potentially important role in pathogenesis.

The haemolytic activity of P. haemolytica is an important characteristic which distinguishes it from P. multocida, another species which causes pneumonia in sheep and cattle. Whether P. haemolytica actually produces a true extracellular haemolysin has not however been proven and its role in pathogenesis is uncertain.

### 1.3. A description of the diseases of sheep caused by P. haemolytica.

#### a) Pneumonia.

The first description of ovine pneumonic pasteurellosis was probably given by Montgomerie et al., (1938), who described the disease as an ovine enzootic pneumonia. Biotype A strains are responsible for pneumonic pasteurellosis in sheep of all ages, but in lambs under two months of age a septicaemic form of the disease is common (Gilmour, 1980). Many healthy sheep are carriers of P. haemolytica and in one survey, biotype A strains were the predominant isolates (94%) from the nasopharynx while 65% of isolates from the tonsils were of the T biotype Gilmour et al. 1974). Studies by Al-Sultan (1982) showed that colonisation occurs soon after birth, and Shreeve and Thompson (1970) found that young lambs reared with their dams acquired an increasing number of serotypes as they grew older whereas lambs removed from their dams harboured few typable strains. This may suggest that early colonisation may occur by intimate contact with the suckling ewe.

Not all carriers however develop pasteurellosis, and evidence for predisposing or concurrent factors such as environmental stress (Harris, 1974) or infections caused by chlamydia (Dungworth and Cordy, 1962; Biberstein et al., 1967), Parainfluenza type 3 (PI3) virus (Hore et al., 1968; Davies et al. 1980; Sharp et al. 1978), adenovirus (Belak et al. 1976; Davies et al. 1980), sheep pulmonary adenomatosis (Sharp and Martin, 1983) and mycoplasma (Jones et al., 1978; Gilmour et al., 1979) in its aetiology have been reported as a result of both field and experimental studies.



In Britain, most outbreaks of pneumonia occur between April and July, with a peak appearance in May and June (Gilmour, 1980). In affected flocks deaths can occur within 24 to 48 hours of the start of an outbreak but mortality seldom exceeds 10% (Gilmour, 1980). Other animals may show high temperatures (up to 42°C), tachypnoea or dyspnoea, coughing and nasal and ocular discharges may be obvious. Recovering animals may develop chronic lung lesions and may be unthrifty thereafter.

Major pathological findings at necropsy include consolidated pneumonic lesions usually of the apical lobes, pericarditis and pleurisy, including a greenish gelatinous exudate and often large volumes of straw coloured pleuritic fluid. Histologically, the major features are alveolar necrosis, interlobular septae are oedematous and lymphoid cell infiltration. A pathognomonic feature of consolidated lesions is the presence of spindle-shaped pyknotic cells called "oat-cells" which form swirls in inter-septal spaces and are generally considered to be degenerate mononuclear cells (Dungal, 1931; Downey, 1957; Stamp and Nisbet, 1963), although some workers have suggested that in bovine pasteurellosis they may be dead neutrophils (Slocombe *et al.*, 1985). Large numbers of *P. haemolytica* can generally be recovered from lesions and exudates and in more chronic cases of the disease *Mycoplasma ovipneumoniae* is also often isolated concurrently (Jones *et al.*, 1978).

#### b) Systemic disease.

The systemic form of ovine pasteurellosis was first described by Stamp *et al.* (1955) in Scotland and was later described in the USA (Biberstein and Kennedy, 1959). The disease is distinct from the pneumonic form in that it involves the T serotypes of *P.*

haemolytica, it causes death only in weaned lambs and is associated with the movement of sheep onto fresh pasture or turnips and rape (Stamp et al. 1955; Suarez-Guemez, 1985). Prodromal signs are rarely observed, and mortality can be as high as 20% in a flock. Afflicted live animals are generally moribund and have a frothy oral discharge. In this disease lung lesions are always present, the entire lungs being distended and the air passages filled with frothy fluid. Lesions can also be found in the liver, spleen, alimentary tract, oesophagus and sometimes the kidneys. Large numbers of organisms are usually recovered from these sites and fewer numbers may also be isolated from the blood.

#### 1.4. Experimental systems and models of infection with

##### P. haemolytica.

##### a) Pneumonia.

In order to test the efficacy of experimental vaccines against pneumonic pasteurellosis it was necessary to develop an experimental system in sheep, or a model in laboratory animals in which to reproduce the natural disease. Problems associated with the experimental infection of conventionally-raised sheep were 1) P. haemolytica is an ubiquitous commensal of the upper respiratory tract (Gilmour et al., 1974) 2) normal animals usually have serum antibodies to P. haemolytica derived from either prior exposure to P. haemolytica or perhaps other bacteria containing cross-reactive antigens. These factors may explain the general resistance of sheep to experimental infection that has been observed previously (N. Gilmour, personal communication).

However, Biberstein et al. (1967) using pulmonary intubation produced a non-lethal disease in 43% of conventionally-reared animals which was similar in its pathology to the natural disease. The proportion of animals susceptible to this infection was increased with a predisposing chlamydial infection, indicating the possible importance of synergistic microbial infections in pneumonic pasteurellosis. Jones et al. (1978) also successfully infected conventional lambs with P. haemolytica after pre-disposition with mycoplasma infection. The pneumonia produced, however, was not the classical enzootic pneumonia described for the majority of field cases of pneumonic pasteurellosis (Montgomerie et al., 1938) but had the characteristic pathology of a chronic pneumonia and was termed "atypical pneumonia" (Gilmour et al., 1979).

Gilmour et al. (1975), using hysterectomy-derived, colostrum deprived lambs which were fed on sterilised milk and concentrates and designated specific pathogen free (SPF) lambs produced pneumonia in 40% of infected lambs. This was later increased to 90% when lambs were predisposed with PI3 virus seven days before an aerosol of P. haemolytica (Sharp et al., 1978). This has since become the accepted method for consistent experimental reproduction of ovine pneumonic pasteurellosis at the Moredun research institute.

The use of SPF lambs for experimental infection and vaccine studies is however costly and dependent upon the seasonal availability of lambs. Because of this many attempts have been made to develop small animal models of P. haemolytica infection. However it has been found that P. haemolytica is non-pathogenic in various laboratory animals including cats, dogs, hens, pigeons,

rabbits and guinea pigs (Dungal, 1931; Lovell and Hughes, 1935; Beveridge, 1937 and Montgomerie *et al.*, 1938). *P. haemolytica* is also of low pathogenicity in mice, but Smith (1958) developed an intraperitoneal (IP) model of infection which used mucin to increase virulence. This simple model only recorded death or survival and was improved upon by Evans and Wells (1979), who enumerated bacteria in the liver of infected animals. The latter model was used successfully to demonstrate significant protection in vaccinated mice. However, one objection to the mouse model and one which Evans herself alluded to (Evans, 1979) was that it had no resemblance to the mode of natural infection, and that the immunological response elicited in the peritoneum was likely to differ from that generated in the lung.

Differences in the immunological response of the mouse to *P. haemolytica* compared to the sheep have also assumed much more importance with the finding that a cytotoxin produced by *P. haemolytica*, which is specifically lethal for leucocytes of ruminants (Benson *et al.*, 1978; Sutherland *et al.*, 1983 Richards *et al.*, 1982), is not toxic for mouse leucocytes (A.D. Sutherland, unpublished findings). In view of these shortcomings, the use of a mouse model for ovine pasteurellosis was not attempted in this thesis.

#### b) Systemic pasteurellosis.

Reproduction of systemic pasteurellosis in sheep with T biotype *P. haemolytica* has not yet been entirely successful. Inoculation of  $1 \times 10^{9.9}$  colony forming units per ml ( $\text{cfu ml}^{-1}$ ) of type T organisms caused death in adult sheep (Smith, 1960) but this number of organisms was only marginally lower than the number of killed

organisms required to cause death suggesting that death was caused by endotoxaemia alone.

Suarez-Guemez et al. (1985) produced systemic T biotype disease in sheep treated with hydrocortisone and in which dietary alterations had induced acidosis. Hydrocortisone is immunosuppressive and the value of this system for studying vaccination is therefore dubious. Attempts to predispose animals to disease by inducing acidosis alone failed (N. Gilmour, Personal communication), perhaps indicating the necessity for immunosuppression in this system. The absence of a reliable and acceptable method for the reproduction of systemic pasteurellosis currently hampers progress in vaccination and pathogenesis studies.

#### 1.5. Vaccination against P. haemolytica serotype A in lambs.

Doubts about the efficacy of commercial vaccines against pneumonic pasteurellosis have been expressed (Gilmour et al., 1979; Gilmour, 1983; ADAS., 1984) and have prompted several suggestions as to why these vaccines may be ineffective. For example, infections in flocks may be due to serotypes not included in the vaccines, the antigens in these vaccines may not be appropriate for protection or they may not be presented in the best manner or with the most efficient adjuvant.

With many developments culminating in a successful experimental infection system for reproducing pneumonic pasteurellosis in SPF lambs (Sharp et al., 1978) the efficacy of vaccines to combat this disease could be tested. In 1983, Gilmour et al. showed that serotype specific protection could be obtained against pneumonic pasteurellosis in SPF lambs using sodium salicylate extract (SSE)



vaccines. These SSE vaccines gave significant homologous protection against serotypes A1, A6 and A9 but were poorly protective against the A2 serotype which required the addition of heat killed organisms to SSE vaccines to give reasonable protection. Also, there was no heterologous protection observed with SSE vaccines (Gilmour et al., 1979).

The poor protection against the A2 infection by SSE vaccines was worthy of particular note since this serotype has been shown in two surveys to be the most prevalent isolated from field cases of the disease (Thompson et al., 1977; Fraser et al., 1982b). Because of this poor protection against serotype A2 and a lack of heterologous protection, research on vaccines has continued to search for improvements in these areas.

One central hypothesis to the development of new effective vaccines against the A2 serotype was that previous vaccines did not contain the correct protective antigens and that if these could be identified and incorporated into vaccines, protection against A2 infection would be possible. In order to prove that it was possible to protect against A2 infection, Donachie et al. (1986a) treated lambs experimentally infected with P. haemolytica with antibiotics as soon as they showed signs of disease. These "convalescent" lambs were then exposed to a second challenge of PI3 virus and P. haemolytica A2 (Sharp et al., 1978). These lambs were highly immune to the second challenge, thus proving that immunity to serotype A2 was possible. Additionally, serological analysis indicated that some humoral immune mechanisms correlated with protection while cell-mediated immunity (CMI) was not stimulated. These findings were in contrast to those of Wells et

al. (1979), who concluded that since passive transfer of immune serum failed to give protection against P. haemolytica infection CMI was probably of more importance than humoral immunity. An alternative conclusion in view of the findings of Donachie et al. (1986a) might support the idea that for antibodies to be protective they must be raised against the correct antigens.

#### 1.6. The interaction of effector mechanisms of immunity with P. haemolytica.

##### a) Mechanisms of immunity in the lung.

Non-specific innate barriers (Table 1.2) provide the initial resistance of the host to microbial invasion of the mucosal surfaces including the lung. However, the ability of specific effector-mechanisms of immunity to combat the establishment of infection by microbial pathogens is of more importance in terms of vaccine strategy since these, once primed, can retain immunological "memory" which responds when infection is encountered. Immunity to extracellular bacterial pathogens such as P. haemolytica is generally recognised as being mediated by humoral immunity whereas protection from intracellular pathogens such as Listeria monocytogenes and Salmonella typhimurium is more dependent upon CMI and especially on the T-lymphocyte activation of macrophages (Sharma and Remington, 1980). Since P. haemolytica is an extracellular pathogen, only the mechanisms of immunity related to infection by extracellular pathogens will be considered here.

In recent years the lung has been recognised as having a distinct mucosal immune system which may be independent in part from the systemic immune system. This mucosal immune system was



Table 1.2 Antibacterial non-specific defence mechanisms at mucosal surfaces

1. Surface	Mechanical barrier Factors in secretions (mucus, lysozyme, lactoferrin) Gastric acidity
2. Mechanical elimination	Ciliary movement Coughing Peristalsis Micturition
3. Phagocytic cells	Granulocytes, monocytes and macrophages
4. Soluble factors	C-reactive protein Fibronectin Transferrin/lactoferrin The alternative complement pathway Mucus Lysozyme

termed the bronchus-associated lymphoid tissue (BALT) in line with the term gut-associated lymphoid tissue (GALT) (Bienenstock *et al.*, 1973). These mucosal immune systems provide local CMI, and locally synthesised secretory IgA (sIgA), IgG and IgM contribute to local humoral immunity (Tomasai and Bienenstock, 1968; Tomasai, 1976). IgG is the predominant class of immunoglobulin in the lower regions of the sheep lung (Gorin *et al.*, 1979) while IgA dominates the upper respiratory tract (Watson and Lascelles, 1971). Transudation of immunoglobulin and other factors from serum may also contribute to local immunity. Wells *et al.* (1977a) found that radio-labelled IgG1 and IgG2 were transported from serum to sheep nasal secretions. Passively acquired IgG to PI3 virus was detectable in nasal secretions of colostrum fed lambs (Smith, 1975; Smith *et al.*, 1976) and passive transfer of immune serum protected swine against mycoplasmal pneumonia (Lam and Switzer, 1971) and cattle against bovine pleuropneumonia (Masiga *et al.*, 1975). Wells *et al.* (1977b) suggested that passively acquired maternal IgG represented an important humoral defence at the mucosal surfaces prior to active production of IgA and IgM.

In the lower lung the alveolar macrophage is considered initially to be the major means of particle clearance and facilitates the inactivation of infectious microorganisms (Reynolds and Newball, 1976) and generally it is thought that the phagocytic macrophage can eliminate small amounts of bacteria which enter the lung. However, during active infection or inflammation polymorphonuclear leucocytes (PMNL) and monocytes (MO) may be recruited to the lung to augment the phagocytic potential of the resident macrophage pool (Davies and Penwarden, 1981).

Attraction of PMNL and MO to the lung occurs by the release of chemotaxins or chemotaxinogens. Chemotaxins stimulate chemotaxis directly and may be host derived factors such as the C5 des arg complement fragment (Fernandez et al., 1978) and prostaglandin E or bacterial products (Keller and Sorkin, 1967; Ward et al., 1968; Schiffmann et al., 1975). Chemotaxinogens indirectly cause chemotaxis usually by complement activation. Bacterial chemotaxinogens include LPS (Morrison and Ulevitch, 1978) and peptidoglycan (Verbrugh et al., 1980).

PMNL are more active in phagocytosis of bacteria than are mononuclear cells (Steigbigel et al., 1974) and once internalised, bacteria can be attacked by hydrolytic enzymes (Spitznagel and Schafer, 1985) and microbicidal oxygen intermediates (Beaman and Beaman, 1984) leading to intracellular killing and degradation. PMNL can also secrete degradative enzymes such as lysozyme, and cationic proteins (Weiss et al., 1978) which can cause extracellular killing (Pryzwansky et al., 1979).

Phagocytosis of particles, including bacteria, can be enhanced by opsonins. The complement component C3b can attach directly to bacterial surfaces or can combine with antibody bound to surface antigens. Professional phagocytes have special receptors on their surfaces for C3b which promote the more rapid and increased attachment of the opsonised particle (Rabinovitch, 1975).

In the immune animal, immunoglobulins, the Fc region of which can be recognised by receptors on the phagocyte membrane, also act as opsonins. Silverstein et al. (1963) identified four classes of immunoglobulins in the sheep by immunoelectrophoresis. These were, IgG1 (fast 7S $\gamma$  or 7S $\gamma$ 1), IgG2 (slow 7S $\gamma$  or 7S $\gamma$ 2), IgM ( $\beta$ 2M or  $\gamma$ 1M)

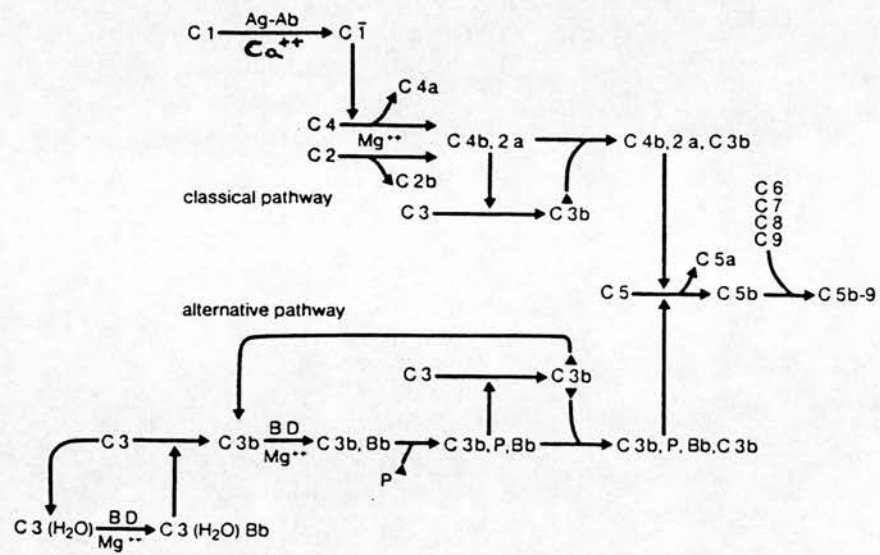
and IgA ( $\beta$ 2A or  $\gamma$ 1A). Since then a subclass of IgG1 (IgG1a) (Curtain, 1969) and IgG3 (Babel and Lang, 1976) have been described. The presence of IgD (Heimer et al., 1969) and IgE (Pan et al., 1968) has also been reported. Watson (1976) has found the sheep IgG2 subclass of immunoglobulin to be a major opsonin, while in the human, only IgG1 and IgG3 are opsonic (Griffin et al., 1977). IgA has been generally considered to be non-opsonic and also unable to fix complement. IgM is also poorly opsonic but unlike IgA it binds complement very efficiently and may promote opsonisation via C3b receptors.

Glass et al. (1981) have also shown the presence of "lectin-like" receptors on phagocyte membranes which can specifically bind bacterial surface ligands to promote phagocytosis independently of opsonins. Binding of S. albus to these "lectin-like" receptors was inhibited by 10 mM D-galactose which may have been an analogue of the bacterial surface ligand.

Complement can be directly bactericidal for many Gram-negative bacterial species by virtue of the membrane attack complex (C5b to C9). This complex can be activated via the alternative or the classical complement pathways (Fig. 1.3.). The role of antibodies in activating the classical pathway probably makes this the most important method of stimulating bactericidal activity with regards to actively-acquired immunity, since it can be manipulated by vaccination with suitable antigens.

Little evidence is available to indicate that complement mediated bactericidal activity operates at mucosal surfaces. Physiological conditions in the gut and urinary tract are unlikely to support complement and the activity of complement in the lung is

Fig. 1.3. Diagram of the classical and alternative pathways of complement activation.





largely undetermined. Jonas and Broad (1972) found a sheep lung washing did not cause haemolysis of antibody-rabbit red blood cell complexes. However, the lung washing could restore the haemolytic activity of sera which were depleted of individual complement components, suggesting that these factors were present in the lung wash. Complement consists of a number of labile components and it is perhaps possible that some of these did not survive the processing of the lung washing. Burrells (1986) found that C3 acted as an acute-phase reactant when levels increased in the lungs of sheep infected with PI3 virus and P. haemolytica. It is possible that complement factors, like immunoglobulins, are transudated into the lung during active infection. The role of complement as an acute-phase reactant has been reviewed by Atkinson and Frank (1980).

Besides being opsonins and mediating bactericidal activity, immunoglobulins can directly neutralise and inhibit the function of bacterial antigens and virulence factors. Since it is not opsonic and does not combine with complement, the major anti-bacterial activity of sIgA appears to be inhibition at the mucosal surface (Williams and Gibbons, 1972). Agglutination, inhibition of motility and of toxins are also attributed to immunoglobulin function at the mucosal surface.

b) Perturbation of ovine immune mechanisms by P. haemolytica.

Amongst pathogenic bacteria there are a plethora of cellular and extracellular pathogenic determinants which can cause perturbation of host defence mechanisms. These pathogenic determinants have been the subject of many monographs, reviews, colloquia and books (Smith, 1977; Steele et al., 1977; O'Grady and Smith, 1980;

Easmon et al., 1983 and Falcone et al., 1984) which show that each pathogenic species is equipped with pathogenic determinants and that strain virulence is usually associated with the expression of one or more of these determinants in which case they are termed strain virulence factors (Smith, 1977).

Serotypes of *P. haemolytica* express a number of virulence factors which may be involved in a) stimulation b) avoidance / inhibition or c) destruction of host effector mechanisms of immunity. These factors include 1) capsule, 2) LPS, 3) cytotoxin, 4) haemolysin, 5) protease(s) and 6) neuraminidase. It is uncertain, however, what role if any each of these factors may play in pathogenesis and of what value they may be as vaccine antigens.

Capsule: the presence of a hyaluronic acid capsule has been associated with avoidance of phagocytosis by *P. multocida* type A strains opsonised with immune and non-immune sera (Maheswaran and Thies, 1979; Anderson et al., 1984). However, the presence of antibodies directed against the capsular antigens of strains of other bacteria such as *Streptococcus pneumoniae* (Giebink et al., 1977) and *E. coli* (Van Dijk et al., 1979) has been associated with an increase in phagocytosis due to opsonisation. As early as 1945, Macleod et al. found that capsular vaccines were protective against capsulate serotypes I, II, IV and VII of the pneumococcus and natural protection has been associated with opsonic antibodies directed against capsular antigens (Bateman and Rowley, 1969) suggesting that phagocytosis involving anti-capsular antibodies is important in protection.

Extrapolation from these studies indicates that while the antigenically specific capsules of *P. haemolytica* serotypes may be involved in the avoidance of phagocytosis in the non-immune animal, they may also be capable of stimulating protective opsonic antibodies if included in vaccines.

SSE vaccines were found to be protective against homologous *P. haemolytica* A1, A6 and A9 infections but not against heterologous challenge (Gilmour *et al.*, 1983). Protection appeared to be associated with anti-capsular antibodies, in that IHA titres were raised by vaccination with the A1 vaccine which gave homologous protection, but an A2 SSE vaccine which was poorly protective did not stimulate IHA titres. Correlation of IHA titre with the response to challenge by individual animals was however not successful. It can be speculated that the A1 SSE vaccine may have increased opsonophagocytosis by stimulating anti-capsular antibodies. This would also have accounted for the serotype specific protection observed.

The serotype A2 capsule is poorly immunogenic in mice (Evans and Wells, 1979), rabbits and sheep (Gilmour *et al.*, 1979) and this may account for the poor protection given by serotype A2 SSE vaccines. However, "convalescent" lambs produced by Donachie *et al.* (1986a) were immune to a subsequent homologous A2 infection and immunity was associated with opsonic antibodies detected in their sera and lung washings.

Capsules have also been implicated in inhibition of phagocytosis and complement-mediated bacterial killing by masking somatic antigens. For example, although staphylococcal capsules do not prevent binding of opsonins to cell-surface antigens (Peterson *et*

al., 1978), they do however prevent increased phagocytosis by inhibiting the interaction of opsonins with phagocyte receptors (Wilkinson et al., 1979). MacDonald et al. (1983) however showed that a bovine isolate of serotype A1 P. haemolytica was susceptible to the bactericidal effects of the classical complement pathway, but no attempts were made to correlate this activity with protection against disease.

LPS: biotype A serotypes are believed to possess rough type LPS by their elution characteristics on gel-filtration columns (Donachie et al., 1984b). In contrast, the T4 serotype has a smooth type LPS (Perry and Babiuk, 1984). P. haemolytica LPS has been shown to have many of the pathophysiological effects of endotoxin (Keiss, 1964; Rimsay et al., 1981). Brogden et al. (1984) found that P. haemolytica A1 LPS, when injected into the sheep lung elicited a biphasic leucocyte response typical of endotoxin. LPS instillation also induced an influx of neutrophils into the lung, suggesting that P. haemolytica LPS may be a chemotaxinogen. Because of these findings, LPS has been considered to be important in the pathogenesis of pneumonic pasteurellosis. Protection against pasteurellosis might therefore include the production of antibodies which can inhibit endotoxin. LPS has also been found to be an important antigen in complement mediated bactericidal activity against Gram-negative bacteria (Glynn and Ward, 1970; Tramont et al., 1974). Donachie et al. (1984b) found that PWE vaccines containing LPS could protect mice against experimental P. haemolytica challenge.

Cytotoxin: this is probably the most important virulence factor of P. haemolytica. The P. haemolytica cytotoxin is a bacterial

cell-free toxin (an exotoxin) that specifically kills leucocytes of sheep (Sutherland et al. 1983; Sutherland, 1985), cattle (Benson et al., 1978; Maheswaran et al., 1980; Walker et al., 1980; Markham and Wilkie, 1980; Berggren et al., 1981; Shewen and Wilkie, 1982a) and goats (Chang et al., 1982; Richards et al., 1982) but not those of non-ruminants (Sutherland et al., 1983). The apparent specificity of *P. haemolytica* cytotoxin for ruminant leucocytes suggests the requirement for a specific receptor for the mediation of toxicity. That the cell types affected are of the naturally infected hosts of *P. haemolytica* lends further support to the idea that this leucocidin is an important pathogenic determinant of this organism.

A notable feature of experimental pneumonic pasteurellosis in sheep is the consistent recovery of high numbers of *P. haemolytica* from the lungs of animals in the early phases of disease (Sharp et al., 1978; Rushton et al., 1979; Davies and Penwarden, 1981). Proliferation of *P. haemolytica* may be a consequence of functional impairment of alveolar macrophages by the cytotoxin. Even at sub-lethal doses the cytotoxin has been shown to inhibit the production of chemotactic factors by bovine alveolar macrophages (Markham et al., 1982). The importance of neutrophil chemotactic response in pasteurellosis is suggested by the findings of Davies and Penwarden (1981), who noted that the clearance of *P. haemolytica* from the lungs of infected sheep was coincident with the influx of PMNL. Neutrophils are more avid in phagocytosis of bacteria than macrophages and bovine neutrophils have been found to be less susceptible to cytotoxin than macrophages (Markham et al., 1982). These may be essential attributes which allow recruited



PMNL to clear P. haemolytica from the infected lungs of sheep and cattle.

Cytotoxic activity can be neutralised by antibodies produced in calves by vaccination with live P. haemolytica (Baluyut *et al.*, 1981), and several necropsy studies have correlated resistance to bovine pasteurellosis with antibody titres to cytotoxin (Shewen and Wilkie, 1982b; 1983a; Cho *et al.*, 1984; Gentry *et al.*, 1985a). Shewen and Wilkie (1983b) also showed cross-neutralisation of the cytotoxin produced by twelve serotypes of P. haemolytica with rabbit antisera raised against them. This extracellular toxin is unlikely to have been present in previous cell-extract vaccines used experimentally and commercially against ovine pneumonic pasteurellosis, and therefore has the potential to be a novel antigen with possible cross-protective capacity.

Haemolysis: attempts have been made to determine whether P. haemolytica cytotoxin is responsible for the haemolysis displayed by this organism on sheep blood agar. Culture supernatants with leucocidal activity were not, however, haemolytic for sheep or cattle red blood cells (Baluyut *et al.*, 1981). This work therefore questioned whether P. haemolytica produces an extracellular haemolysin.

Lui (1959) demonstrated that haemolysis produced by V. cholera on blood agar was not due to a true haemolysin but rather to lysis of red blood cells by alkaline conditions brought about by colony growth. Paradoxically, production of a true haemolysin was shown to occur at pH 7.0. It is possible that haemolysis by P. haemolytica is also attributable to the effects of bacterial fermentation. However, a role for haemolysis in pathogenesis has



been suggested by the finding that injection of haemoglobin with P. haemolytica enhances infection in mice (Chengappa et al., 1983; Al-Sultan and Aitken, 1984). This increased disease presumably by making iron more readily available for bacterial growth. On the basis of these studies, further work to identify the cause of haemolysis by P. haemolytica and the possible role this attribute has in pathogenesis and immunity seems merited.

Protease: P. haemolytica produces an extracellular neutral protease (Otulakowski et al., 1983) which attacks sialoglycopeptides. These authors speculated that this protease may contribute to virulence by either unmasking receptors for cytotoxin on macrophage membranes or by cleaving a possible proform of the cytotoxin.

Neuraminidase: Tabatabai and Frank (1981) first described the presence of a neuraminidase in the supernatant of P. haemolytica A1 cultures which was inhibited by antibodies raised against it in rabbits. Neuraminidase production has since been observed in the majority of other serotypes (Frank and Tabatabai, 1981; Otulakowski et al., 1983), but only the latter authors found neuraminidase to be produced by the A2 serotype.

All of the above virulence factors may be important antigens against which various mechanisms of immunity may contribute to the elimination of infection. Consequently, in the formulation of effective vaccines it may be important to include a number of these antigens. In a multifactorial vaccine approach, many mechanisms of immunity may be stimulated which may work in synergy or individually if circumstances dictate to eradicate infection.

### 1.7. Aims of the thesis.

This thesis formed part of a project to determine the antigens and mechanisms of immunity involved in protection against ovine pneumonic pasteurellosis caused by serotype A2 *P. haemolytica*. The aim of the work was to identify and characterise antigens and virulence factors of *P. haemolytica* which can modulate host mechanisms of immunity and are therefore of potential importance in vaccine formulations. At the outset, the hypothesis was formed that the production of cytotoxin neutralising (CN) antibodies by vaccination may contribute to protection against ovine pneumonic pasteurellosis. CN antibodies may help to prevent disease at the earliest stages of infection by increasing bacterial clearance, due to reduced phagocytic cell death (which may also prevent adverse inflammatory responses), and increasing lymphocyte responsiveness. It was however considered that cell-surface antigens would be required in effective vaccines to induce bacterial clearance by enhanced opsonophagocytosis or by stimulating antibody-activated complement-mediated bacterial killing.

It was considered that factors of particular importance were:

- 1) characterisation of *P. haemolytica* cytotoxin and an assessment of the value of a cytotoxin preparation as a vaccine antigen
- 2) an assessment of the role of serum CN antibodies and opsonophagocytosis in immunity
- 3) the susceptibility of *P. haemolytica* serotypes to serum killing.

The role of this effector mechanism of immunity in disease pathogenesis was also examined.

## SECTION 2

Materials and Methods

## 2.1. Bacterial strains.

Unless otherwise stated, the following *P. haemolytica* serotype strains were used throughout this thesis: serotype A1, strain V1763; serotype A2, strain T884; serotype T3, T635; serotype T4, R317; serotype A5, T683; serotype A6, D0133; serotype A7, D0800; serotype A8, C53; serotype A9, D0775; serotype T10, D0770; serotype A11, D0759h; serotype A12, N498; serotype A13, D0804; serotype A14, 22.7.87; serotype T15, D0685; serotype A16, 26.10.87. Strains were stored at  $-70^{\circ}\text{C}$  in nutrient broth (Oxoid) in 0.5 ml volumes and thawed as required.

2.2. Production of *P. haemolytica* cytotoxin.

Cytotoxin was expressed in supernatant fluids of dialysis sac cultures and prepared as described by Sutherland (1985). Dialysed and lyophilised supernatant fluid, termed crude cytotoxin, was prepared as described by Sutherland and Redmond (1986).

See flow chart at the end of Section 2.

2.3. Preparation of *P. haemolytica* A2 SSE and LPS.

The SSE and LPS were prepared from *P. haemolytica* organisms as described by Donachie *et al.* (1984b).

See flow charts at the end of Section 2.

## 2.4. Polyacrylamide gel electrophoresis (PAGE).

## 2.4.1. Sodium dodecyl sulphate (SDS)-PAGE.

The SDS-PAGE was performed on 10% gels by the method of Laemmli, (1970) using a slab-gel apparatus (Bio-Rad, U.K.) and a power pack (Pharmacia Ltd, Milton Keynes, U.K.). Samples were run at 13 mA

limiting current per gel until the dye-front reached the bottom of the gel . After electrophoresis gels were stained with either silver or Coomassie blue dye by the methods of Morrissey (1981).

#### 2.4.2. Detection of proteases by PAGE.

Proteases were detected in *P. haemolytica* serotype A1 and A2 crude cytotoxin preparations by a modification of the method of Heussen and Dowdle (1980) for detecting enzyme activities in SDS-PAGE gels containig suitable substrates. Samples (1 mg) of crude cytotoxin were resuspended in: a) SDS-PAGE solubilising buffer which did not contain 2-mercaptoethanol. The samples were not heated before loading onto gels b) normal SDS-PAGE solubilising buffer and the samples were heated at 100°C before loading. Samples were loaded onto 10% SDS-PAGE gels containing 0.1% azocasein (Sigma, U.K.) which was incorporated into gels as a substrate. Casein was found to be a digestible substrate for protease activities in crude cytotoxin preparations by Otulakowski *et al.* (1983). Samples were run at 13 mA per gel as described above. When electrophoresis was completed gels were washed in 2.5% aqueous Triton X100 (Sigma) for 1 hour at room temperature. Gels were then rinsed in distilled water three times and incubated in 0.1 M glycine pH 8.3 buffer for 48 hours at room temperature. Proteases were shown by negative staining in which digested areas of substrate were detected in gels stained in Coomassie blue dye (Morrissey, 1981).

#### 2.4.3. Detection of LPS in proteinase K digests by SDS-PAGE.

Proteinase K (Sigma) digests of P.haemolytica whole cells (5 mg ml<sup>-1</sup>) were prepared by the method of Hancock and Poxton (1988) and the structure of the LPS of various serotypes of P.haemolytica was examined by running digests on SDS-PAGE. Gels (10%) were prepared on a midget-gel apparatus (LKB, Sweden.) and loaded with 10 µl of each digest. Gels were run at a constant voltage (100 V) until the dye-front reached the bottom of the gel. Gels were stained with silver by the method of Tsai and Frasch (1982) to visualise LPS.

#### 2.5. The protocol for inducing "convalescence" in SPF lambs.

The protocol for inducing "convalescence" in SPF lambs was described by Donachie et al. (1986a). Table 2.1 outlines the experimental protocol used to induce "convalescence" in group 1 SPF lambs while the other (group 2) lambs remained untreated. These groups were then experimentally challenged with parainfluenza type 3 (PI<sub>3</sub>) virus followed by homologous P. haemolytica A2 strain as indicated in Table 2.1.

Fig. 2.1. SDS-PAGE of affinity chromatography purified IgG (arrows) from a "convalescent" lamb serum. lane 1, 10  $\mu$ l of IgG; lane 2, molecular mass standards.

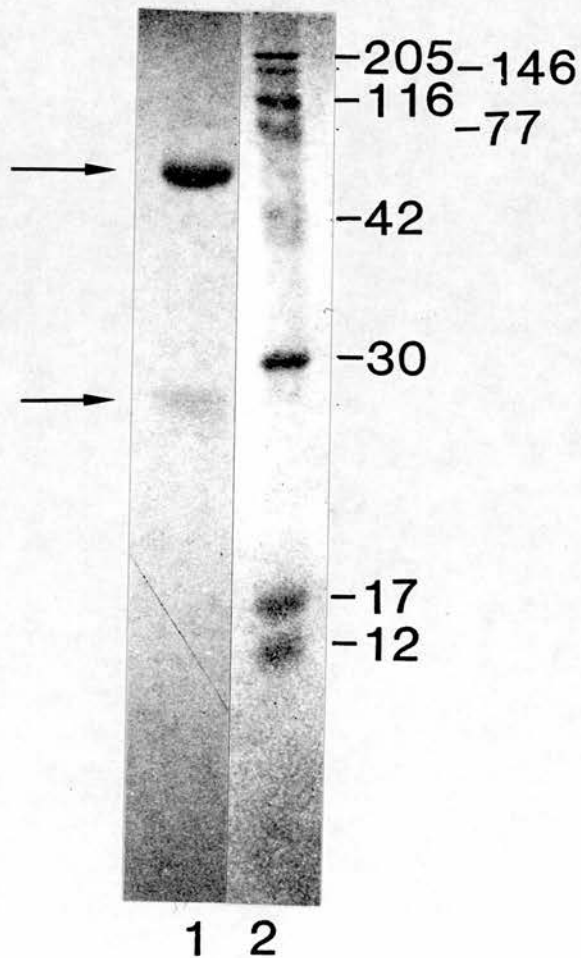


Table 2.1. Experimental protocol to produce "convalescence" in SPF lambs (see text p 38).

<u>Group (n)</u>	Infection with <u>P. haemolytica</u> A2 (day 0)	Infection with PI3 virus (day 21) and <u>P. haemolytica</u> A2 (day 28)	Necropsy (day 35)
1 (7)	+	+	+
2 (7)	-	+	+

+ = treated

- = untreated



## 2.6. Purification of IgG from "convalescent" immune serum.

Sheep IgG was purified from a "convalescent" lamb serum by affinity-column chromatography. The column was a pig anti-sheep IgG (Fab<sub>2</sub>) ligand bound to Sepharose 4B (Pharmacia, U.K.) and was donated by Mr. A. Dawson. A volume (10 ml) of "convalescent" lamb serum (at 2.27 g IgG L<sup>-1</sup>) was loaded onto the column. Loading of the serum and elution of the IgG was done as described in the affinity chromatography handbook (Pharmacia). The desorbed IgG fractions (60 ml total volume) were pooled and dialysed overnight against 0.05 M Tris/HCl buffer pH 7.0. The IgG fraction was then concentrated and exchanged with phosphate-buffered saline pH 7.2 (PBS) on a collodion bag apparatus (Sartorius, U.K.) and finally adjusted to an IgG concentration equivalent to that in the original whole serum (2.27 g l<sup>-1</sup>). The IgG was shown to be purified by running a sample (10 µl) on SDS-PAGE (12.5% gel) along with 5 µl of molecular mass markers composed of Sigma type G8511 and M3889 plus BDH type 44261 2F markers, reconstituted as recommended. The IgG was identified as consisting of a 53 kDa heavy chain and a 26 kDa light chain (Fig 2.1.).

## 2.7. Immunoblotting of *P. haemolytica* antigens.

Dot blotting of *P. haemolytica* antigen preparations was performed by adding 2.0 µl volumes of the antigen preparation to strips of BA 83 nitrocellulose paper (Schleicher and Schuell, Dassel, W. Germany) and allowing the antigen to air-dry. Western blotting of antigens resolved on SDS-PAGE gels was performed by the method of Burnette (1981) as modified by Herring and Sharp (1984).

After the antigens were bound to nitrocellulose, the nitrocellulose was blocked in 50% FBS in wash buffer (PBS pH 7.2 containing 0.5% Tween 20 (Sigma), 0.037% EDTA and 2.5% Na Cl) for 1

hour at 37°C and then washed three times quickly in wash buffer followed by three 10 min washes in wash buffer. The blocked nitrocellulose was then incubated at 37°C for 1 hour in wash buffer containing a 1 in 200 dilution of donkey anti-sheep IgG conjugated with horse radish peroxidase (HRP) (Scottish Antiserum Production Unit, Law hospital, Carlisle).<sup>\*</sup> The nitrocellulose was again washed three times quickly and three times for 10 mins in wash buffer. Blots were developed by adding the substrate (5 mg of diaminobenzidine) (Sigma) together with 20  $\mu$ l of H<sub>2</sub>O<sub>2</sub> in 20 ml of substrate buffer (0.1M Tris/HCl pH 7.4) and developing for 5 mins. Development was stopped by running the blot under tap water.

#### 2.8. Statistical analysis.

All analysis was done using the "Minitab" statistical computer-programme package (Minitab, State College, Pennsylvania, USA.). Significance testing between grouped data was done by the two-sample T-test unless otherwise stated. Correlations between data were calculated using the Spearman rank correlation coefficient test.

\* In Section 4, before the addition of anti-sheep IgG conjugate, specific T10 sera were added to the blot at a 1 in 50 dilution in wash buffer. The blot was incubated in the sera at 37°C for 1 hour, washed three times quickly and three times for 10 mins in wash buffer. In Section 5, the addition of exogenous specific antibody to the blot was omitted.

Flow chart of the production of P. haemolytica crude cytotoxin by dialysis sac culture

Defrost 0.5 ml aliquot of P. haemolytica from  $-70^{\circ}\text{C}$  and inoculate into 50 ml of nutrient broth (NB). Incubate  $37^{\circ}\text{C}$  overnight (static)



Pellet bacteria (5,000 g for 30 mins), wash x1 in phosphate buffered saline pH 7.2 (PBS)



Resuspend bacterial pellet in 400 ml of PBS and inoculate 20 ml volumes into the lumen of sterile dialysis sacs (9 inch) sealed at one end and suspended in 500 ml of NB. Incubate on a shaker at 100 rpm at  $37^{\circ}\text{C}$  for the required period.



Collect sac contents and pellet bacteria (5,000 g for 30 mins). Filter sterilise supernatant fluid through a  $0.45\ \mu\text{M}$  membrane. Place supernatant fluid in a dialysis sac and dialyse exhaustively (several changes over 36 hours) against distilled water at  $4^{\circ}\text{C}$ .



Lyophilise dialysate (crude cytotoxin) and weigh.

Flow chart of the production of P. haemolytica cell wall sodium salicylate extract (SSE).

Defrost 0.5 ml aliquot of P. haemolytica from  $-70^{\circ}\text{C}$ . Inoculate into 50 ml of nutrient broth (NB). Incubate  $37^{\circ}\text{C}$  overnight (static).



Inoculate 30 ml of culture into 3 l of NB, incubate  $37^{\circ}\text{C}$  for 6 hours on an orbital shaker at 100 rpm. Pellet bacteria at 5,000 g for 30 mins and resuspend in 300 ml of 1M sodium salicylate. Shake suspension at  $37^{\circ}\text{C}$  for 3 hours.



Pellet bacteria (30,000 g for 30 mins at  $4^{\circ}\text{C}$ ) and dialyse supernatant fluid against phosphate buffered saline pH 7.2 (PBS) for 48 hours.



Concentrate dialysate to 30 ml by ultrafiltration on a YM 100 membrane (Amicon)



Dialyse retentate against distilled water for 48 hours at  $4^{\circ}\text{C}$  and lyophilise.

Flow chart of the production of P. haemolytica lipopolysaccharide (LPS).

Defrost 0.5 ml aliquot of P. haemolytica from  $-70^{\circ}\text{C}$ . Inoculate into 50 ml of nutrient broth (NB). Incubate  $37^{\circ}\text{C}$  overnight (static).



Inoculate 30 ml of culture into 3 l of NB, incubate  $37^{\circ}\text{C}$  for 18 hours on an orbital shaker at 100 rpm. Pellet bacteria at 5,000 g for 30 mins and resuspend in 50 ml of distilled water (DW).



Warm bacteria suspension to  $68^{\circ}\text{C}$  and add to 50 ml of 90% aqueous phenol at  $68^{\circ}\text{C}$ . Maintain at  $68^{\circ}\text{C}$  for 10 mins.



Remove the mixture and cool on ice until phase separation occurs. Centrifuge at 5,000 g for 30 mins at  $4^{\circ}\text{C}$ .



Collect upper (aqueous) phase and dialyse for 36 hours against running tap water. Lyophilise.



## SECTION 3

Detection and Some Characterisation of *P. haemolytica* A2 Cytotoxin and Cytotoxin Neutralising Antibody.

Live organisms of all the known serotypes (1 to 15) of *P. haemolytica* have been shown to be cytotoxic for ovine macrophages (Sutherland and Donachie, 1985). Culture supernatant fluid from serotype A1 was also found to be lethal for ovine macrophages (Sutherland *et al.*, 1983), PMNL and lymphocytes obtained from peripheral blood, and gastric duct derived lymphocytes (Sutherland, 1985), but not tissue culture cells or leucocytes from other non-ruminant species (Shewen and Wilkie, 1982a; Sutherland *et al.* 1983)

Toxicity is therefore attributable to an extracellular factor which has specificity for ruminant leucocytes including those of cattle (Shewen and Wilkie, 1982a) and goats (chang *et al.*, 1982; Richards *et al.* 1982) as well as sheep. As such, the cytotoxin is apparently active only against leucocytes of the natural hosts of *P. haemolytica*. This restricted activity may be a reason for the host-range specificity of this pathogen, and since it is lethal for cells involved in immunity, the toxin is likely to be an important pathogenic determinant.

Because of its virulence attributes the cytotoxin, if antigenic, may also have potential as a vaccine component. Since protection against serotype A2 infection has remained particularly elusive, emphasis was accorded here to the production and characterisation of cytotoxin by this serotype with a view to using this antigen in a novel vaccine preparation. However, since most

of the current knowledge of the *P. haemolytica* cytotoxin has been derived from serotype A1 cultures and some of the physical and chemical characteristics of the toxin have been described (Baluyut *et al.* 1981; Sutherland and Redmond, 1986), the A1 serotype toxin was used as a standard with which the serotype A2 toxin was compared.

A crude cytotoxin preparation from serotype A1 *P. haemolytica* was previously derived from nutrient broth dialysis-sac culture supernatant fluid (Sutherland and Redmond, 1986). The advantages of this technique were that it was cheaper than culture in RPMI medium supplemented with foetal bovine serum (FBS), which was used by others (Markham and Wilkie, 1980; Baluyut *et al.* 1981), and that after dialysis, bacterial products (crude cytotoxin) were free from contaminating broth components. This technique was therefore used in these studies for the production of serotype A2 crude cytotoxin.

Cytotoxicity was first detected by Benson *et al.* (1978), who reported morphological changes in bovine macrophage monolayers inoculated with live *P. haemolytica* organisms. The lethality of the extracellular cytotoxin for ruminant leucocytes was later examined either by using a trypan blue exclusion assay (Baluyut *et al.* 1981 ; Sutherland *et al.* 1983) or by a method involving the release of chromium-51 isotope from labelled bovine leucocytes (Markham and Wilkie, 1980) which was modified to a microtitre plate format by Shewen and Wilkie, (1983a). Since the technique involving chromium-51 labelling was more sensitive than the trypan

blue exclusion assay it was adapted here for the detection of cytotoxicity using ovine bronchoalveolar macrophages (BAM) as target cells.

3.1. Detection of *P. haemolytica* serotype A1 and A2 cytotoxin in dialysis-sac culture supernatant fluids obtained after 6, 18 and 24 hours.

Dialysis sac culture (1 ml, produced as described in Materials and Methods) was collected on three separate occasions at 6, 18 and 24 hours from cultures of *P. haemolytica* A1 and A2. Samples were centrifuged for 5 min on a microfuge (MSE, Fisons Instruments, Crawley, UK.) at 13,000 g to pellet bacteria. The culture supernatant fluid was filter-sterilised through a 0.45  $\mu$ m filter (Millipore, UK.) then stored at  $-70^{\circ}\text{C}$  until assayed for toxicity. Toxicity was assayed by mixing duplicate 200  $\mu$ l volumes of culture supernatant fluid with chromium-51 labelled ovine BAM in a microtitre-plate.

Sheep BAM for the cytotoxicity assay were isolated as described previously by Burrells (1986), then resuspended at  $4 \times 10^7$  viable BAM  $\text{ml}^{-1}$  in RPMI 1640 medium (Imperial Laboratories, U.K.) supplemented with 10% heat-inactivated foetal-bovine serum (Gibco, U.K.), 1% glutamine, 3 mM HEPES buffer, 2% sodium bicarbonate and adjusted to pH 7.4 with 1M Na OH (complete RPMI medium). BAM counts and viability were assessed by trypan blue exclusion (0.4% dye in PBS) in an improved Neubauer haemocytometer. Sodium chromate (chromium 51-isotope) (Amersham, U.K.) was added to the BAM suspension at 50  $\mu\text{Ci ml}^{-1}$  and incubated at  $37^{\circ}\text{C}$  for 1 hour, after which cells were sedimented and washed three times in Hanks'

balanced salt solution (Hanks' BSS) by centrifugation at 100 g for 10 min at room temperature. The BAM suspension was finally resuspended in complete RPMI medium at  $2 \times 10^6$  viable BAM  $\text{ml}^{-1}$ .

The toxicity of culture supernatant fluids for sheep BAM was assessed by adding duplicate 200  $\mu\text{l}$  volumes of each preparation to wells of sterile, tissue-culture grade, flat-bottomed microtitre plates (NUNC, Denmark). Then BAM (100  $\mu\text{l}$ ) were added to each well, including spontaneous release control wells, containing 200  $\mu\text{l}$  of complete RPMI medium, and total release control wells, containing 200  $\mu\text{l}$  of Triton X 100 detergent (Sigma). Plates were sealed with sterile, plastic, adhesive film (Flow Laboratories, Irvine, Scotland) and incubated at  $37^\circ\text{C}$  for 3 hours. BAM were then sedimented by centrifugation of the plates at 100 g for 10 min at  $4^\circ\text{C}$ , after which 100  $\mu\text{l}$  of supernatant fluid was removed from each well for counting in a gamma-isotope counter (type auto-gamma 5650, Canberra Packard, U.K.). The percent specific toxicity for each supernatant was calculated from the formula:

$$\text{percent specific cytotoxicity} = 100 \times \frac{\text{counts in sample} - \text{counts in spontaneous release control}}{\text{counts in total release control} - \text{counts in spontaneous release control}}$$

The chromium-51 release assay was found to be faster, more economical in materials and more sensitive than the trypan blue exclusion assay used by this author previously (Sutherland *et al.*, 1983) and gave reproducible results from duplicate samples. Test results (Table 3.1) showed that there was no significant difference in the levels of toxicity given by three separate batches of supernatant fluid prepared from each of the 6, 18 and 24 hour cultures.

Table 3.1. The percentage toxicity of three separate batches of serotype A1 and A2 crude cytotoxin from 6, 18 and 24 hour cultures

Culture period (hours)	<u>Percentage specific cytotoxicity<sup>a</sup> for:</u>					
	Serotype A1 preparations			Serotype A2 preparations		
	1	2	3	1	2	3
6	70	73	71	66	42	63
18	67	73	73	61	40	59
24	68	83	72	69	20	71

<sup>a</sup> Percentage specific cytotoxicity =  $100 \times \frac{\text{counts in sample} - \text{counts in spontaneous release control}}{\text{counts in total control} - \text{counts in spontaneous release control}}$

In view of these results, 18 hours was chosen as the standard culture period for cytotoxin production since, it was, 1) the most convenient for working with 2) it was considered that this preparation may contain more cytotoxin antigen than earlier cultures which, although it may not be biologically active, may be antigenic and therefore of value in vaccine preparations.

### 3.2. Some characteristics of the antigens present in the crude cytotoxin preparation.

A previous study (Sutherland and Redmond, 1986) found that a serotype A1 crude cytotoxin preparation consisted largely (90 %) of polysaccharide while only a small percentage of protein was present. The polysaccharide was not characterised, however, it is possible that it consisted of capsular and lipopolysaccharide antigens. Evidence for the presence of these antigens in serotype A2 crude cytotoxin was therefore sought with the A1 toxin being included for comparison.

#### 3.2.1. SDS-PAGE analysis (see Materials and Methods).

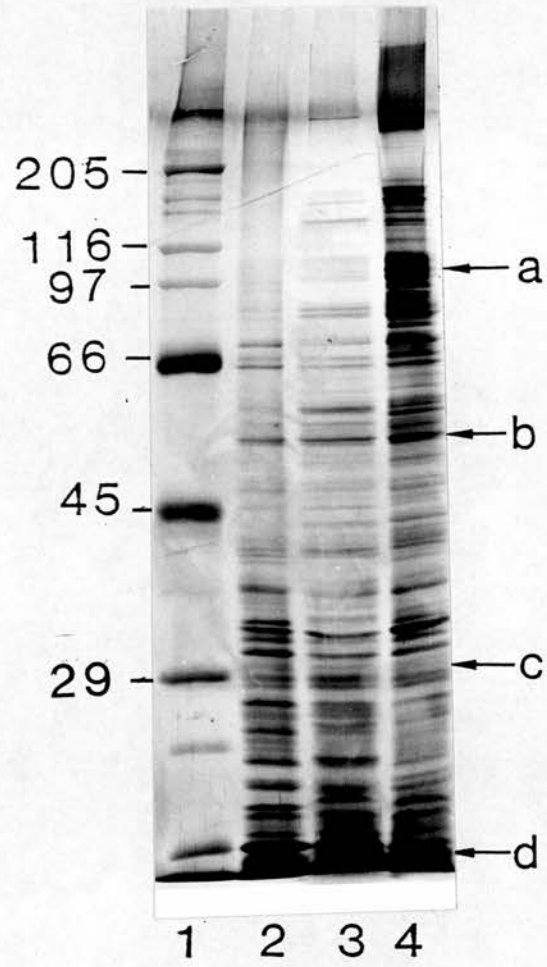
Lyophilised serotype A1 and A2 crude cytotoxin and A2 SSE cell-surface antigens (each prepared as described in the Materials and Methods) were resuspended in phosphate buffered saline pH 7.2 (PBS) and mixed 1:1 with 2X SDS-PAGE solubilising buffer. A sample of serotype A1 (400 µg) and A2 (800 µg) crude cytotoxin preparations, A2 SSE (10 µg) and 5 µl of SDS-6H molecular weight standards prepared as described by the manufacturer (Sigma), were loaded into wells of a 10% SDS-PAGE gel. The gel was silver stained by the method of Morrissey (1981) and photographed.



The SDS-PAGE profile (Fig 3.1) of the serotype A1 cytotoxin was essentially the same as that previously described (Sutherland and Redmond, 1986). The profile of the polypeptide bands in the A2 serotype cytotoxin was also similar to that of the A1 cytotoxin, but there was quantitatively less of each band. Both serotype A1 and A2 toxins contained distinctly resolving antigen bands which were of equivalent molecular mass to some antigens present in the SSE. These were therefore considered to be OMPs, including the major OMPs which are 51 kDa (arrow b) (Donachie *et al.* 1984b), and 29 kDa (arrow c) (Squire *et al.*, 1984) in molecular mass. When gel loading concentrations were considered, the outer membrane antigens found in the toxin preparations appeared to be about 40 times less in quantity compared to the SSE. LPS was tentatively identified in the cytotoxin preparations as a broad band running at the gel dye-front (arrow d). High molecular mass polysaccharide antigen (possibly capsule) was also seen as a diffuse indistinct area of staining at the upper end of the gel.

Bands unique to the cytotoxin preparations (not in SSE) were identifiable between 96 and 116 kDa and were more prominent in the A1 toxin preparation (arrow a). These bands were tentatively identified as cytotoxin antigens since recent work by Lo *et al.* (1986), with a recombinant serotype A1 cytotoxin (leucotoxin) expressed in *E. coli*, identified a 102 kDa band as the cytotoxin protein, and Chang *et al.* (1987) identified a 105 kDa cytotoxin antigen which degrades to 95 kDa during culture.

Fig. 3.1. Silver stained SDS-PAGE gel of:  
lane 1, molecular mass standards (5  $\mu$ l); lane  
2, serotype A2 sodium salicylate extract of  
cell-surface antigens; lane 3, A2 crude  
cytotoxin, lane 4, serotype A1 crude cytotoxin.

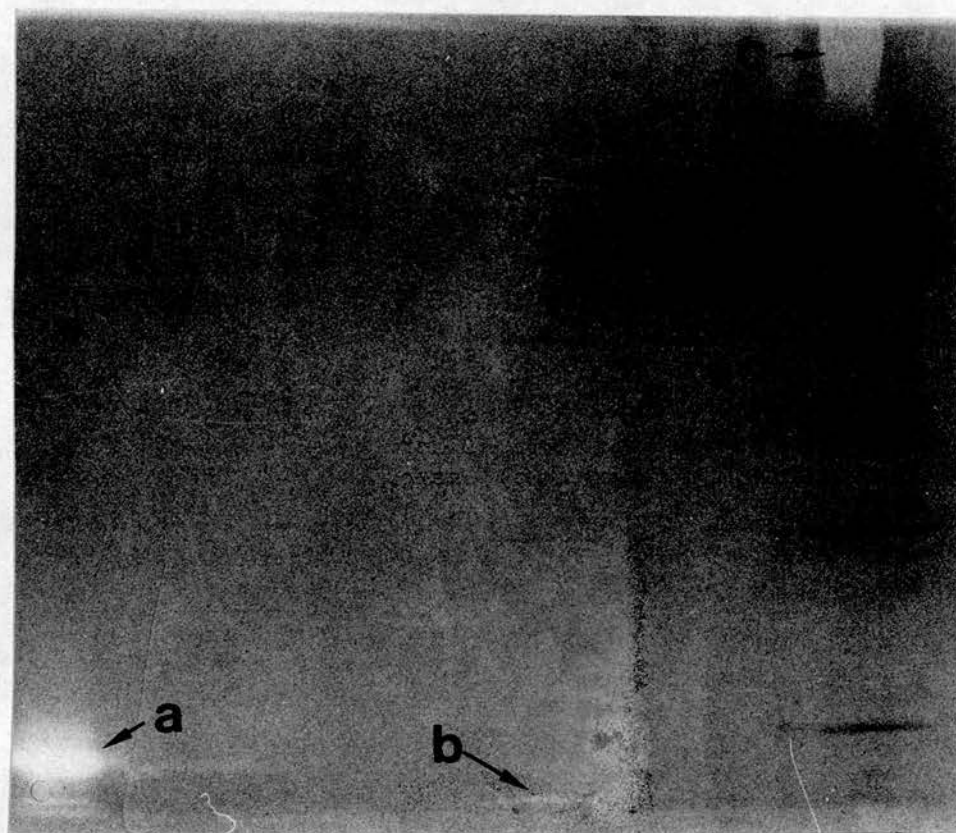


### 3.2.2. Identification of protease activity in crude cytotoxin preparations.

Otulakowski et al. (1983) found that protease activity was present in A1 crude cytotoxin preparations. These authors however did not attempt to identify the antigens responsible for this activity. Heussen and Dowdle (1980) have developed a method for detecting plasminogen activators by incorporating the zymogen and gelatin substrate in SDS-PAGE gels and negatively staining the digested areas of gelatin around the resolved activator bands. This method was used to attempt detection of proteases in crude cytotoxin preparations. Otulakowski et al. (1983) had found that the proteases could digest casein and so 0.1% azocasein was used as a substrate for incorporation in gels. The method is fully described in the Materials and Methods. The relative mobilities (Rf values) of proteases were calculated by the method of Hames and Rickwood (1984).

Areas of proteolytic activity were detectable in Coomassie blue stained SDS-PAGE gels containing azocasein (Fig 3.2). The A1 crude cytotoxin had a large protease (Rf 0.05) which was located at the top of the gel (arrow c) while the A2 toxin preparation had a less distinct and smaller (Rf 0.99) protease which ran at the dye-front (arrow b). Chymotrypsin A4 (Boehringer Mannheim) was included in the gel as a positive protease control (arrow a) and had a major proteolytic band with an Rf value of 0.95. The smaller sized (Rf 0.99) protease in the A2 toxin preparation was found to be labile to heating at 100°C for 5 mins in solubilising buffer containing 2-mercaptoethanol, whereas the larger sized protease (Rf 0.05)

Fig. 3.2. SDS-PAGE showing negatively-stained areas of protease activity: lane 1 (arrow a), chymotrypsin (50  $\mu$ g); lane 2 (arrow b), serotype A2 crude cytotoxin (1.0 mg); lane 3 (arrow c), serotype A1 crude cytotoxin (1 mg).



1

2

3





found in the A1 toxin preparation was not (results not shown). This suggested that these proteases were unlikely to be related isomers.

### 3.2.3. Detection of LPS in serotype A2 crude cytotoxin.

A modified limulus amoebocyte lysate (LAL) assay (G. Moon and C. Hodgson, in preparation) was used to confirm the presence of LPS, which was tentatively identified in the SDS-PAGE gel profile of the crude A2 cytotoxin (part 3.2.1.). The assay was based on the observation by Levin and Bang (1968) that the interaction of LAL with endotoxin (LPS) produces a turbidity which increases with reaction time. The kinetics of turbidity were followed spectrophotometrically using a centrifugal analyser (IL Multistat III plus, Instrumentation Laboratories).

Logarithmic dilutions (ranging from a concentration of 10,000 down to  $1.0 \text{ pg ml}^{-1}$ ) of a standard LPS preparation (*E. coli* 0113, H10, K<sup>-</sup>, Atlas Bioscan), and the crude cytotoxin sample  $7.0 \text{ mg ml}^{-1}$ , were added manually (120  $\mu\text{l}$ ) to sample wells and 40  $\mu\text{l}$  of LAL (Pyrogell GT, Atlas Bioscan) was added to the reagent wells of the analyser rotor. The LAL and LPS samples were allowed to equilibrate at  $37^{\circ}\text{C}$  for 3 mins before optical density (OD) readings were taken at 380 nm wavelength. When the OD of the LAL/LPS mixtures reached an OD which remained steady over a period of 20 to 30 secs this OD was taken as the baseline reading. From this baseline, the time it took for each concentration of the standard LPS preparation to give a rise in OD of 0.02 (the onset time) was calculated. The onset time for each LPS concentration was then plotted on log-log graph paper to produce a standard

curve. The onset time for the crude cytotoxin sample was plotted on the standard curve and the LPS concentration calculated by extrapolation.

The assay detected LPS at  $1 \times 10^6$  endotoxin units  $\text{mg}^{-1}$  in the crude A2 cytotoxin preparation, where  $1 \mu\text{g}$  of the standard LPS was equivalent to 10 endotoxin units.

#### 3.2.4. Detection of serotype-specific capsular antigen in crude cytotoxin.

Confirmation of the presence of the serotype-specific capsular antigen in serotype A1 and A2 crude cytotoxin was sought by titrating the cytotoxin in the IHA test (Biberstein *et al.*, 1960) against 1 in 20 dilutions of standard serotyping antisera raised in rabbits at the Moredun Research Institute.

The serotype A1 cytotoxin had capsular antigen which was titratable down to  $10.0 \mu\text{g ml}^{-1}$ , while the serotype A2 cytotoxin had antigen which was titratable down to  $40.0 \mu\text{g ml}^{-1}$ . The capsular antigen in each cytotoxin did not cross-react with the antiserum of the other serotype, thus confirming the specificity of the capsular antigen.

#### 3.3. Standardisation of activity in crude cytotoxin preparations by titration.

In order to use cytotoxin preparations in a cytotoxin neutralisation (CN) assay for comparing the CN titres of sera from day-to-day and from different experiments it was first considered necessary to standardise the cytotoxin preparations for activity. Standardisation of the toxicity of each preparation by titration

was also intended to ensure the quality control and repeatability of toxin production.

Crude cytotoxin preparations from serotypes A1 and A2 (100  $\mu$ l volumes) were titrated in duplicate, in doubling dilutions, from 20  $\text{mg ml}^{-1}$  down to 0.01  $\text{mg ml}^{-1}$ . Each dilution was overlaid with 100  $\mu$ l of medium to replace the test serum which would be added in the CN assay. Finally, 100  $\mu$ l of chromium 51 labelled BAM were added to all assay wells and the assay carried out as described in part 3.1. The toxicity end-point for each toxin preparation was taken to be at the lowest dilution which gave toxicity of 10% or greater, and was expressed in  $\text{mg ml}^{-1}$ .

Twelve batches of serotype A1 crude cytotoxin were found to have a mean toxicity end-point of 0.12 ( $\pm$  0.05)  $\text{mg ml}^{-1}$ , while twenty-one batches of serotype A2 crude cytotoxin had a mean toxicity end-point of 8.1 ( $\pm$  1.4 )  $\text{mg ml}^{-1}$  (Table 3.2 ). Serotype A1 therefore produced significantly more ( $p < 0.001$ ) detectable toxin than serotype A2.

Crude cytotoxin from a further four ovine serotype A 2 field isolates (F1, 2, 3 and 4) and one bovine serotype A1 isolate (Fb 1) gave toxicity end-points which were within the range of those given by their respective standard strains (table 3.3). This indicated that the lower toxicity of serotype A2 crude cytotoxin compared to the A1 toxin was a consistent attribute within the serotype and was conserved amongst isolates.

Crude cytotoxin derived from four earlier log-phase (6 hour) dialysis sac cultures of both serotype A1 and A2 gave mean toxicity end-points of 0.04 and 3.4 ( $\pm$  0.94)  $\text{mg ml}^{-1}$  respectively (Table 3.4), which were not significantly different from the levels of

Table 3.2. Toxicity end-points of batches of crude cytotoxin from serotypes A1 and A2 18 hour cultures

<u>Toxicity end-point (mg ml<sup>-1</sup>) of:</u>		
	<u>serotype A1</u>	<u>serotype A2</u>
	0.04	5.0
	0.63	2.5
	0.16	10.0
	0.16	10.0
	0.04	5.0
	0.08	5.0
	0.08	2.50
	0.04	0.16
	0.04	1.25
	0.04	0.16
	0.04	10.0
	0.04	5.0
		5.0
		5.0
		10.0
		10.0
		20.0
		10.0
		20.0
		10.0
		20.0
<hr/>		
Mean	0.12 ( $\pm$ 0.05)	8.1 ( $\pm$ 1.4)
( $\pm$ standard error)		

Table 3.3. Toxicity end-points for crude cytotoxin from field isolates of *P. haemolytica*

<u>Toxicity end-points (mg ml<sup>-1</sup>) for field isolates</u>				
Serotype A2				Serotype A1
F1	F2	F3	F4	Fb1
5.0	20	20	10	0.3

Table 3.4. Toxicity end-points of crude cytotoxin from serotypes A1 and A2 6 hour cultures

Toxicity end-point (mg ml <sup>-1</sup> )		
	<u>Serotype A1</u>	<u>Serotype A2</u>
	0.04	2.5
	0.04	5.0
	0.04	5.0
	0.04	1.25
Mean (± standard error)	0.04	3.4 (± 0.94)

activity detected in the 18 hour homologous cultures. This suggested that the difference observed in the levels of extracellular cytotoxin found between the A1 and A2 serotypes was not due to differences in the time of release of the toxin between the serotypes.

#### 3.4. Development and use of an assay to detect CN antibodies.

Shewen and Wilkie (1983a), Cho et al. (1984) and Gentry et al. (1985a, 1985b) have correlated the presence of CN antibodies with resistance of cattle to pneumonic pasteurellosis. The possibility existed therefore that CN titres might be associated with immunity in sheep protected against P. haemolytica. To investigate this possibility, sera and lung washings from "convalescent" lambs (Donachie et al., 1986a) were compared with those of naive SPF lambs for CN activity. This study might be expected to provide experimental evidence for the involvement of CN in immunity against ovine pneumonic pasteurellosis.

Stimulation of CN antibodies in rabbits by vaccination with serotype A2 crude cytotoxin was also attempted to give preliminary information on whether this type of preparation may be useful as an experimental vaccine component.

##### 3.4.1. Detection of CN antibodies in the sera and lung washings of "convalescent" SPF lambs.

The protocol for inducing "convalescence" in SPF lambs was described by Donachie et al. (1986a) and is included in Materials and Methods (table 2.1).

Sera tested for CN titre were taken from "convalescent" lambs



(group 1) prior to the primary *P. haemolytica* infection (day 0) and from group 1 and group 2 (untreated control) lambs immediately before the secondary infection (day 28). Lung washings were taken at necropsy from all survivors. Titres of CN antibodies (Table 3.5) in the sera and lung washings of experimental animals were assessed by titrating samples in duplicate against crude cytotoxin preparations which were used at a four-fold concentration of the toxicity end-point (part 3.3). Volumes (100  $\mu$ l) of each sample were titrated in duplicate with complete RPMI medium in flat-bottomed microtitre plates and then 100  $\mu$ l of crude cytotoxin was added to each dilution and left at room temperature for 10 mins to allow antibody-toxin interaction to occur. BAM (100  $\mu$ l) were then added to each assay well and the assay was carried out as described for the cytotoxicity assay in part 3.1. The CN titre of each sample was taken as the highest dilution which neutralised cytotoxic activity by >80% and was calculated from the formula:

$$\text{CN titre} = 100 \times \frac{\text{the percent specific toxicity of the cytotoxin} - \text{the percent specific toxicity of the cytotoxin in the presence of the test sample}}{\text{the percent specific toxicity of the cytotoxin}}$$

No serum CN titres were detectable against serotype A2 cytotoxin in group 1 lambs (day 0) before "convalescence" was induced. CN titres were however, detected in the sera of group 1 lambs on day 28 after the primary infection and these were significantly greater ( $p < 0.001$ ) than those in group 2 lambs which had negligible CN titres. Group 1 lambs also had CN titres in their lung washings which were significantly greater ( $p < 0.05$ ) than the titres in the three group 2 lambs which survived until necropsy.

Table 3.5. Cytotoxin neutralisation (CN) titres in sera and lung washings of convalescent and untreated SPF lambs

<u>Group</u>	<u>animal</u>	CN Titres in:		
		<u>serum</u>		<u>lung washing</u>
		day 0 (pre 1st infection)	day 28 (post 1st infection)	day 35 (necropsy)
Convalescent lambs	1	0	32	16
	2	0	NA	64
	3	0	16	8
	4	0	64	32
	5	0	32	16
	6	NA	64	NA
	7	NA	16	16
	mean ( $\pm$ standard error)	0	37.3 ( $\pm 8.92$ )	25.3 ( $\pm 8.37$ )
Untreated controls	1	NA	0	8
	2	NA	1	8
	3	NA	NA	8
	4	NA	1	NA (died)
	5	NA	0	NA (died)
	6	NA	0	NA (died)
	7	NA	NA	NA (died)
	mean ( $\pm$ standard error)		0.4 ( $\pm 0.25$ )	8.0

NA = not available for testing

NA (died) = sample not available because lamb died before day of necropsy

The three survivors in the group 2 lambs however, had CN titres which may have been associated with their survival and was probably due to a rapid primary response to the secondary infection. However, it was not possible to compare the lung washings of those group 2 lambs which died before necropsy.

Since all the convalescent animals were completely immune to subsequent experimental challenge (Donachie *et al.* 1986a) serum and lung washing CN titres were therefore associated with protection of "convalescent" lambs against further experimentally induced pasteurellosis.

#### 3.4.2. Detection of CN antibodies in a rabbit vaccinated with a serotype A2 crude cytotoxin preparation.

A cytotoxin-based vaccine was prepared by resuspending lyophilised crude cytotoxin in sterile distilled water and adsorbing with sufficient Alhydrogel (Miles) to give a final concentration of  $10 \text{ mg ml}^{-1}$  cytotoxin in 0.27% Alhydrogel solution. A rabbit, which had been pre-bled to ensure that it had no CN antibodies was vaccinated twice subcutaneously (sc) with two doses 28 days apart and bled two weeks after the second vaccination. The vaccine was found to have induced a CN titre of 1 in 4 in the CN assay.

### Discussion

Cytotoxicity against ovine BAM was readily measured in a chromium-51 release assay. Production of serotype A2 cytotoxin in dialysis sac cultures which were grown for 6, 18 or 24 hours resulted in preparations with equally detectable cytotoxic

activity. This result was different from that of Baluyut et al. (1981) who found toxic activity was detectable only in early log phase (1 to 6 hour) cultures. However, those cultures were grown in RPMI medium supplemented with 10% FBS and it is possible that nutrients necessary for bacterial growth were exhausted after this period. Indeed, Gentry et al. (1986) have suggested that FBS in this type of culture serves mainly as a source of protein-bound iron which is essential for toxin production. Dialysis sac culture with nutrient broth for a period of 18 hours was chosen as a convenient, cost-effective method of toxin production which resulted in a product which was free of nutrient broth constituents.

After titration of several batches of serotype A1 and A2 crude cytotoxin in the chromium-51 release assay it was concluded that potent levels of toxin could be consistently produced by the dialysis sac culture method. However, it was also found through titration that serotype A2 produced significantly less detectable cytotoxin than the serotype A1. This finding may have important consequences for vaccine production. If it is assumed that the lower activity is due to less cytotoxin antigen being produced rather than the A2 toxin being innately less active, then the A1 serotype may provide a better source of cytotoxin antigen for vaccines. It has been shown previously by Shewen and Wilkie, (1983b) that cytotoxin produced by serotypes 1 to 12 were cross-neutralisable with specific antiserum. If the cytotoxin were to prove to be a protective antigen (an immunogen) then it may be cross-protective amongst the P. haemolytica serotypes.

SDS-PAGE analysis showed that the crude cytotoxin preparations

contained in addition to cytotoxin many OMP, LPS and capsular antigens. The presence of LPS was confirmed in an endotoxin assay and the serotype-specific capsule was identified by the IHA test. Two species of extracellular protease were demonstrated in the crude cytotoxin preparations. One, with an Rf value of 0.05, was found in the A1 toxin preparation and was heat stable while the other, with an Rf of 0.99, was found in the A2 cytotoxin preparation and was heat labile. Differences in lability probably indicates that these proteases were of different species and not isoenzymes.

Otulakowski et al. (1983) speculated that protease activity may be involved in promoting the activity of the cytotoxin. It would therefore be of interest to determine if the different proteases found in the crude A1 and A2 cytotoxins are responsible for the differences in activity detected in these preparations. Also, the A2 crude cytotoxin contained less detectable amounts of the 105 and 95 kDa cytotoxin proteins, detected by Chang et al. (1987), when compared to the A1 preparation. It may be that this also was a cause for the lower toxin activity detectable in the A2 crude cytotoxin.

Each of the antigens in the crude cytotoxin may be important immunogens and their detection may have important consequences for understanding any immunity raised by vaccines based on the crude cytotoxin.

The first demonstration that lambs could be effectively protected against *P. haemolytica* A2 infection was given when Donachie et al. (1986a) showed that "convalescent" lambs were immune to subsequent homologous serotype A2 challenge. Some of the mechanisms of immunity which may have been responsible for

protection were examined by these authors, but CN antibody titres were not measured. In these studies, the sera of "convalescent" lambs had no serum CN titres prior to primary infection with P. haemolytica. However, after infection CN titres were detectable. In contrast, untreated, susceptible animals had negligible titres. Thus, CN titres would appear to be associated with immunity to experimental ovine pasteurellosis.

All of the "convalescent" lambs had CN titres in their lung washings at the time of necropsy, indicating that local CN antibody was likely to have been involved in immunity. Three untreated lambs which survived until necropsy also had CN titres in their lung washings, but these were significantly lower and may have been due to a rapidly mounted primary response to the challenge infection. The ability of these animals to mount a rapid CN response, although it did not entirely prevent the formation of pneumonic lesions (Donachie et al. 1986a), may have been associated with their ability to survive until the day of necropsy. Unfortunately, lung washings from the untreated lambs which died could not be taken and were therefore not available for comparison.

The serotype A2 crude cytotoxin preparation was shown to induce a CN response after vaccination of a naive rabbit. The demonstration of the ability of this vaccine to stimulate CN titres, and the association of CN activity with immunity to P. haemolytica infection in lambs, indicates that the crude cytotoxin may have potential as a novel vaccine for raising CN antibodies in lambs and studying their role in protection against ovine pneumonic pasteurellosis.



## SECTION 4

The Bactericidal Effects of Serum on *P. haemolytica*.

In studies relating to protection against bacterial disease, it is important to define the mechanisms of immunity which operate against the causative pathogenic organism. It is also desirable to determine which bacterial antigens can stimulate these protective mechanisms by vaccination.

*P. haemolytica* serotype A1 organisms were found by MacDonald et al. (1983) to be susceptible to killing by the classical complement pathway when they were mixed with complement and immune bovine serum. It is possible therefore, that bactericidal mechanisms are similarly involved in the protection of sheep against pasteurellosis and that this immune mechanism could be stimulated by vaccines containing relevant antigens. In view of this, a micro-titre plate assay was developed to detect antibodies which stimulated the classical complement pathway and induced killing of *P. haemolytica* organisms. With a standard source of complement in this assay, it was assumed that the percentage of bacteria killed (%K) would be proportional to the bactericidal capacity of antibodies in the sample. This was considered to be the most important variable to study since levels of relevant antibodies could be raised anamnesticly after vaccination whereas complement could not. The method was used to study bactericidal activity in the sera of "convalescent" and naive SPF lambs which were respectively either resistant or susceptible to experimental

challenge with P. haemolytica (Donachie et al., 1986a). Also an attempt was made to identify bacterial antigens which were targets for bactericidal antibodies.

#### 4.1. Development and optimisation of a microtitre plate bactericidal assay and characterisation of the susceptibility of P. haemolytica A2 to serum bactericidal activity.

If P. haemolytica A2 is susceptible to the classical but not the alternative pathway of complement, as is the A1 serotype (MacDonald et al., 1983), then bactericidal activity will only occur in the presence of both specific antibody and complement. An assay was therefore developed to detect and characterise bactericidal activity against P. haemolytica A2.

A sample (100  $\mu$ l) of P. haemolytica A2 which was stored at  $-70^{\circ}\text{C}$  was thawed and inoculated into 10 ml of nutrient broth (NB). After incubation at  $37^{\circ}\text{C}$  overnight, 200  $\mu$ l of culture was transferred to 10 ml of NB and incubated at  $37^{\circ}\text{C}$  for 3 hours to obtain a log-phase culture, which was then centrifuged at 11,000 g for 5 min at room temperature (Taylor, 1983). The resultant bacterial pellet was washed once in modified barbitol buffer (MBB) (Oxoid, U.K.) to remove residual NB, which has been shown to be anti-complementary (Muschel and Treffers, 1956). The bacterial inoculum was finally adjusted with MBB to the required number of cfu  $\text{ml}^{-1}$ . The actual bacterial number was confirmed by retrospective plate counting on SBA.

Sera from naive SPF lambs or foetal calves were collected and stored for use as complement sources. Each batch of serum was

tested before use for the absence of bactericidal antibodies and the presence of sufficient complement to cause 100% bactericidal activity when used in conjunction with a standard positive control serum.

The assay was performed in 96-well, tissue-culture grade, flat-bottomed micro-titre plates (NUNC, Denmark) into which reactants were simultaneously delivered to 12 wells with a multi-channel pipette. Triplicate assay wells received 20  $\mu$ l of each standard serum or test sample followed by 100  $\mu$ l of bacterial inoculum. Assay suspensions were incubated for 15 min at room temperature to allow antibody-bacterium interaction to occur. Standardised serum complement was then added in 80  $\mu$ l volumes to each assay well, except when evaluating the effect of antibody on *P. haemolytica* A2 in the absence of complement, when 80  $\mu$ l of heat-inactivated FBS was added.

Assay plates were covered with a sterile adhesive plastic film (Flow laboratories, Irvine U.K.) and incubated at 37°C for the required period. Generally, after incubation, 10  $\mu$ l samples of each triplicate suspension were removed with a multi-channel pipette and inoculated onto SBA plates. When bacterial counts were too high for direct plate counting, as when the assay was being evaluated, sequential logarithmic dilutions of each suspension were made in MBB diluent in microtitre plates before counting. After incubation of SBA plates at 37°C overnight, the mean number of cfu ml<sup>-1</sup> was calculated from each triplicate.

The mean percent of bacterial inoculum which was killed (%K) in each suspension was calculated by the formula:

$\%K = 100 \times 1 - (\text{the mean number of cfu ml}^{-1} \text{ after incubation} / \text{the mean number of cfu ml}^{-1} \text{ before incubation})$ . The  $\%K$  for each lung washing was adjusted by a correction factor (Donachie *et al.*, 1986a) to take account of the extent to which each sample had been concentrated before assay.

A convalescent serum was initially selected as a standard positive serum since it gave 100% killing in the presence of complement. A FBS sample was selected as a standard negative serum since it was not bactericidal in the presence of a complement source.

When optimal assay conditions were developed with respect to bacterial concentration and incubation period it was found that the microtitre plate assay could be performed rapidly with little variation in counts between sample triplicates. The standard positive and negative sera gave repeatable results from day to day. The standard positive serum killed 100% of a bacterial suspension containing  $3.4 \times 10^3$  cfu ml<sup>-1</sup>, but only in the presence of complement. No killing occurred when the complement was replaced with heat-inactivated FBS. This bactericidal capacity rapidly diminished with dilution of the standard positive serum, and was abolished at 1 in 32 dilution (Fig.4.1). The standard negative serum was not bactericidal at any dilution (Fig 4.1). The standard positive serum was tested with various concentrations of *P. haemolytica* A2 (Fig 4.2). The killing activity of the serum remained >85% for *P. haemolytica* concentrations between  $3.4 \times 10^3$  cfu ml<sup>-1</sup> and  $3.4 \times 10^5$  cfu ml<sup>-1</sup>, but thereafter decreased rapidly with increasing bacterial concentration. A bacterial concentration of  $3 \times 10^3$  cfu ml<sup>-1</sup> (equivalent to an inoculum of  $6 \times 10^3$  cfu ml<sup>-1</sup>) was therefore selected as optimum.

Fig. 4.1. Titration of the bactericidal activity of a "convalescent" (x—x) and a naive (o—o) specific pathogen-free lamb serum

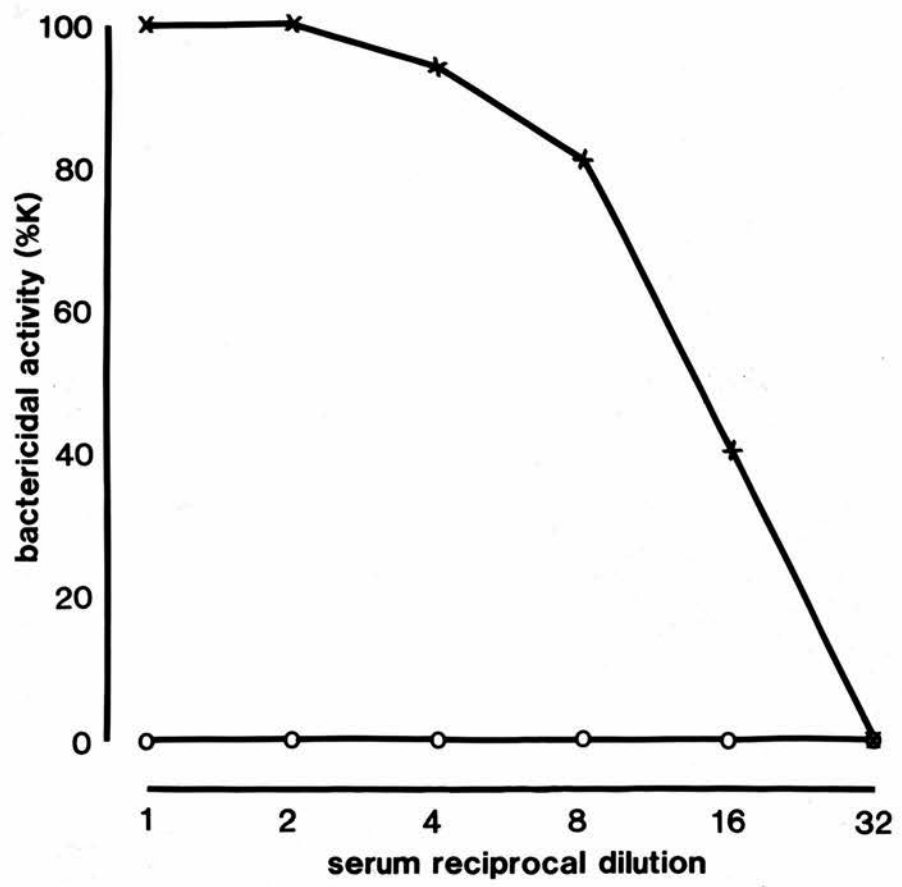
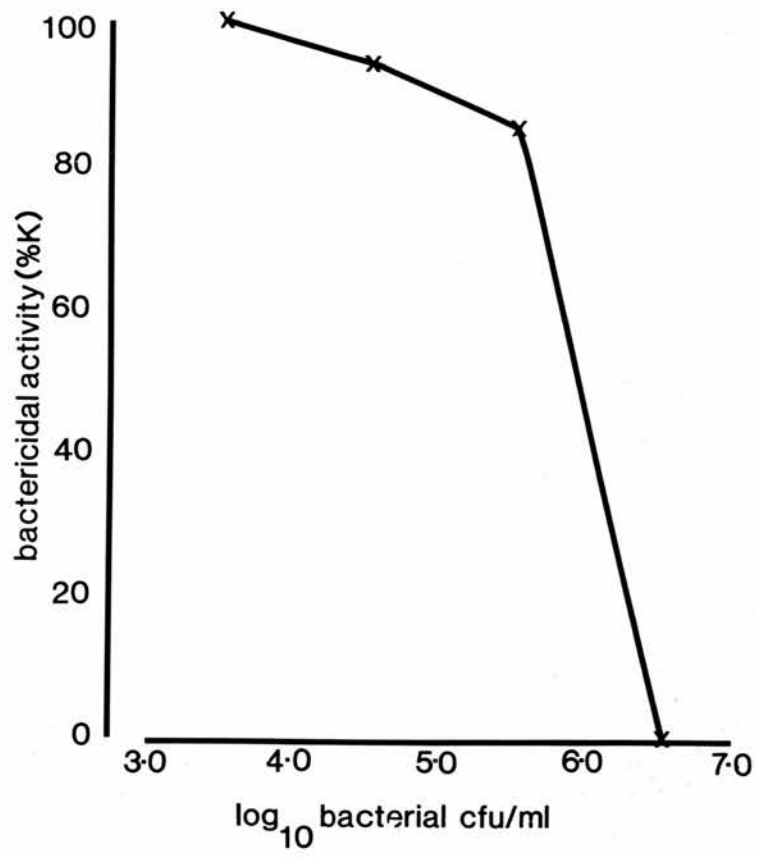




Fig. 4.2. The bactericidal activity of a "convalescent" lamb serum for varying titres of P. haemolytica A2.



Bacterial killing by the standard positive serum was apparent after 2 min of incubation (97%K) and had increased to 100%K by 15 min. An incubation period of 30 min was therefore selected. Fresh FBS was as effective a source of complement as naive SPF lamb serum, but less convenient to obtain. Complement source sera were used undiluted, since the bactericidal capacity of sera decreased rapidly in the presence of a diluted complement source e.g. 100% killing by one serum in the presence of an undiluted complement source was reduced to 86% when the complement source was diluted 1 in 2. Heat inactivation of the complement source completely abolished bactericidal activity, demonstrating that complement was essential. The complement source was not bactericidal in the presence of the standard negative serum, indicating that specific antibody was also essential for bacterial killing. All further assays employed the conditions which were found to be optimal for determining bactericidal activity.

#### 4.2. Bactericidal activity with purified IgG.

Confirmation that antibody was the essential component for bactericidal activity in the standard positive serum was by the use of immuno-affinity purified IgG which was purified from this serum as described in Materials and Methods. IgG proved to be as bactericidal (100%K) as the complete serum when used at the equivalent concentration ( $2.27 \text{ g IgG L}^{-1}$ ).

#### 4.3. Detection of bacterial target-antigens for bactericidal antibodies.

Aliquots of the standard positive serum were absorbed at  $37^{\circ}\text{C}$

for 20 min with  $1 \text{ mg ml}^{-1}$  of either SSE of P. haemolytica A2 cell wall or A2 LPS each of which was prepared as described in Materials and Methods. After absorption, the serum was sterilised by filtration. Absorption with LPS completely abolished bactericidal activity, while the SSE reduced activity to 21% K.

#### 4.4. The susceptibility of four ovine field isolates of P. haemolytica A2 to killing.

Four ovine, serotype A2, field isolates (F1 to F4) were tested for their susceptibility to the bactericidal activity of the standard positive serum to ascertain that the susceptibility of the T884 strain was not unique. The standard negative serum was also tested against each isolate to ensure that these isolates were not susceptible to the alternative complement pathway. The T884 strain was included in the assay as a positive control.

The inocula used in this assay ranged from  $4.6 \times 10^3$  to  $2.0 \times 10^4$  cfu  $\text{ml}^{-1}$ . All four isolates and the positive control strain were completely killed by complement in the presence of the standard positive serum. No killing was observed with the standard negative serum.

#### 4.5. Detection of bactericidal activity in the sera and lung washings of "convalescent" SPF lambs.

If bactericidal activity is important in immunity to P. haemolytica then it is probable that immune animals should have detectable bactericidal antibody levels.

Sera and lung washings from "convalescent" SPF lambs (group 1), which were known to be immune to P. haemolytica A2 infection

(Donachie et al., 1986a), were compared with naive SPF lambs (group 2) for bactericidal activity against P. haemolytica A2. The experimental protocol for the treatment of these lambs is described in the Materials and Methods. The bacterial inoculum used was  $6.4 \times 10^3$  cfu ml<sup>-1</sup>.

Sera taken from the "convalescent" lambs on day 0 (pre-immunising infection) had no bactericidal activity, but those collected on days 34 and 41 had group mean activities of 92.1 and 89.8%K, respectively (Table 4.1). Furthermore, all but one lung washing obtained at necropsy (day 41) from these "convalescent" lambs also contained bactericidal activity, although this was less than in their sera (Table 4.1). Conversely, no bactericidal activity was detected in sera of naive lambs on days 34 and 41 or in lung washings taken from three of this group at necropsy nor were these lambs protected against P. haemolytica A2.

#### 4.6. Studies on the susceptibility of a P. haemolytica biotype T serotype to bacterial killing mechanisms.

Biotype T colonies have a more mucoid colony morphology than A biotype organisms (Gilmour, 1980). Perry and Babiuk (1984) have demonstrated by SDS-PAGE analysis that at least one biotype T serotype (T4) possesses a "smooth" type LPS structure. This type of LPS may contribute to the mucoid appearance of T biotype colonies. Variants of a number of Gram-negative bacterial species possessing "smooth" type LPS have been found to be resistant to complement-mediated bactericidal activity and this resistance is often associated with the ability of variants to cause septicaemia (Turk, 1959; Chedid et al., 1968; Rowley, 1968; Taylor, 1975 and Madonna and Allen, 1981 ).

Table 4.1. The bactericidal activity (%K) of sera and lung washing from "convalescent" lambs<sup>a</sup>

Animal	%K of serum			%K of lung washings <sup>b</sup> (at necropsy)
	Pre-first <sup>c</sup> immunising challenge (Day 0)	Post-first challenge (Day 34)	Post-second challenge (necropsy, Day 41)	
1	0	97.6	95.5	13.2
2	0	NA	98.5	18.0
3	0	93.2	94.5	0
4	0	99.5	99.2	27.0
5	0	99.0	99.6	9.8
6	0	98.0	98.4	11.6
7	0	65.5	42.7	11.3
Group mean ( $\pm$ standard error)	0	92.1 ( $\pm 5.4$ )	89.8 ( $\pm 7.9$ )	12.99 ( $\pm 3.1$ )

<sup>a</sup>Bactericidal activity was not detectable in sera or lung washings of control lambs

<sup>b</sup>The bactericidal activity of lung washings taken at necropsy was adjusted by a correction factor to return all samples to a unit volume of the original harvest

<sup>c</sup>By challenge with live organisms, followed by prevention of fulminating disease by antibiotic therapy

NA - not available



The T biotype serotypes of P. haemolytica cause a systemic disease in young adult sheep (Thompson et al., 1977). Evidence for "smooth" type LPS and associated resistance to bacterial killing might therefore indicate that this is an important pathogenic determinant in the aetiology of the systemic disease. The LPS structure of all the T biotype serotypes was therefore examined by SDS-PAGE and compared with those of the A biotypes.

#### 4.6.1. SDS-PAGE analysis of the LPS structures of 15 serotypes of P. haemolytica.

LPS, prepared by the proteinase K digestion of whole cells (Hancock and Poxton, 1988), was analysed by SDS-PAGE (Materials and Methods). S. minnesota ("smooth" type LPS) and S. minnesota R595 ("rough" type LPS) were included on gels as standards (Fig. 4.3).

The biotype T serotypes (3, 4, 10 and 15) were all found to have a "smooth" type LPS profile, which showed as a typical ladder of polysaccharide bands on the silver stained gels (Tsai and Frasch, 1982). In contrast, the A biotype serotypes 1, 2, 6, 7, 8, 9, 12, 13 and 14 had "rough" type LPS with only the heavily stained core plus lipid A apparent at the bottom of the gels. The A biotype serotypes 5 and 11 were intermediate, having a few faint linear "O"-antigen bands. This study confirmed the finding of Perry and Babiuk (1984) that the T4 serotype had "smooth" type LPS and extended it to include the other T serotypes.

Fig. 4.3. SDS-PAGE of LPS from proteinase K digests of P. haemolytica whole cell preparations. Lanes 1 to 15, A and T biotype serotypes 1 to 15 respectively; lane 16, S. minnesota; lane 17, S. minnesota R595.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

#### 4.6.2. Testing the susceptibility of serotype T10 organisms to bactericidal activity.

Antisera to the serotype T10 were available from lambs which were either made "convalescent" to this serotype or vaccinated with homologous strain crude cytotoxin. Since these immunisation schedules had been found to raise bactericidal activity against serotype A2 organisms (see part 4.5 and section 7) these sera were tested for their bactericidal activity against serotype T10 organisms.

The six anti-serotype T10 sera were raised in sheep. Two were raised in SPF lambs by vaccination with a crude cytotoxin preparation (as described for serotype A2 in section 7), and four in normal adult sheep by live infection with serotype T10 organisms (N. Gilmour, personal communication). The vaccinate sera were taken 12 days after a second vaccination with crude cytotoxin. "Convalescent" sera were taken seven days after a second live infection with serotype T10. All sera were heat inactivated at  $56^{\circ}\text{C}$  for 30 min and stored at  $-20^{\circ}\text{C}$  until used. These sera and the standard negative serum were tested with complement in the bactericidal assay against 100  $\mu\text{l}$  of serotype T10 inoculum ( $7.0 \times 10^3$  cfu  $\text{ml}^{-1}$ ). Serotype A2 (with an inoculum of  $6.2 \times 10^3$  cfu  $\text{ml}^{-1}$ ) was included in the assay as a known susceptible strain and was tested against the standard positive and negative sera.

Serotype T10 organisms were completely resistant to killing (0 %K) in the presence of all sera. The A2 organisms were susceptible to the standard positive serum (100 %K) and resistant to the standard negative serum (0 %K) which indicated that the complement source and assay procedure were functional.

#### 4.6.3. Serological analysis of the anti-T10 immune sera.

Evidence to confirm that the anti-T10 sera were indeed immune was sought by testing them for antibodies to cell-surface antigens in an ELISA and by immunoblotting. The ELISA was performed by the method of Fodor and Donachie (1988), except that the antigen was serotype T10 LPS used at 100  $\mu\text{g ml}^{-1}$  and dispensed in 100  $\mu\text{l}$  per well. Sera were diluted 1 in 100 for use in the ELISA.

Cell-walls of serotype T10 organisms, prepared by the method of Shand *et al.* (1985), were run on SDS-PAGE gels as described in Materials and Methods. Cell wall antigens were transferred to nitrocellulose for immunoblotting with the serotype T10 antisera by the method of Herring and Sharp (1984). Sera were diluted 1 in 50 for immunoblotting.

In the ELISA, the six anti-T10 immune sera gave optical densities (OD) at 495 nm wavelength ranging from 0.14 to 0.63, whereas the standard negative serum gave an OD of 0 (Table 4.2). The immune sera also reacted with cell-wall antigens by immunoblotting, whereas the standard negative serum did not (Fig. 4.4). These results indicated that the immunisation protocols had successfully raised antibodies against T10 cell-surface antigens including LPS.

Table 4.2. Bactericidal and ELISA O.D. of anti-serotype T10 immune sera.

Serum <sup>a</sup>	bactericidal activity v T10 (% killing)	ELISA O.D. <sup>b</sup>
1	0	0.62
2	0	0.14
3	0	0.37
4	0	0.58
5	0	0.61
6	0	0.63
7	0	0.00

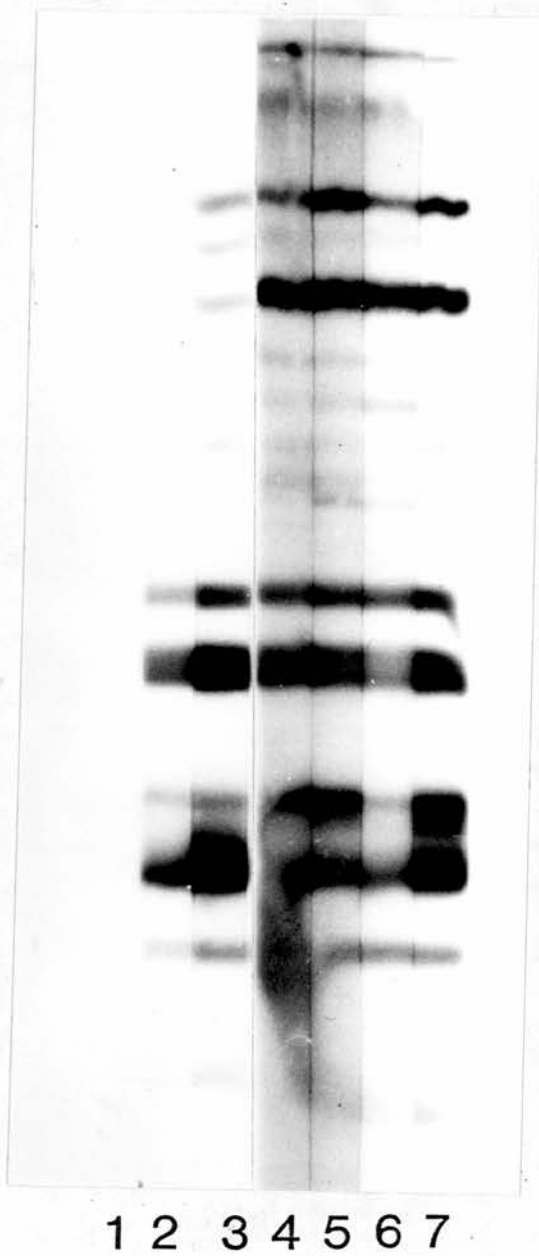
<sup>a</sup>Sera 1 and 2 were from specific-pathogen-free lambs vaccinated with a serotype T10 crude cytotoxin preparation, sera 3 to 6 were from conventionally raised sheep made "convalescent" to infection with serotype T10, serum 7 was the standard negative (SPF lamb) serum.

<sup>b</sup>The maximum possible O.D. was 2.0



Fig. 4.4. Immunoblot of sera reacted against a cell wall preparation of serotype T10 P. haemolytica

Lane 1, is a naive SPF lamb (standard negative) serum; lanes 2 and 3, are post-vaccination sera from two SPF lambs vaccinated with a serotype T10 crude cytotoxin preparation; lanes 4 to 7, are sera from four "convalescent" lambs recovered from a serotype T10 infection.



## Discussion

The bactericidal assay gave little variation amongst replicates and was comparable on a day-to-day basis. Titration of the immune serum and bacterial inoculum indicated that assay results were dependent upon both antigen and antibody titre. The most suitable conditions for assay of SPF lamb sera or lung washings were 20  $\mu$ l of undiluted serum and  $3 \times 10^3$  cfu ml<sup>-1</sup> of serotype A2 P. haemolytica.

Serum complement was not bactericidal for P. haemolytica A2 in the absence of antibody, indicating that P. haemolytica A2 could not activate the alternative complement pathway. This result is in agreement with the findings of MacDonald et al. (1983), who showed that P. haemolytica A1 was not killed in the absence of a functional classical complement pathway. Also, the standard positive serum was not bactericidal unless in combination with complement, indicating that bactericidal activity was due to an antibody-complement complex.

Four field isolates of P. haemolytica A2 were also found to be susceptible to bactericidal activity indicating that the susceptibility of the T884 strain was not unique.

Purification proved that IgG was the bactericidal component in the standard positive serum. The bactericidal activities of IgA and IgM against P. haemolytica A2 were not investigated.

Previous studies have shown that IgM is more active than IgG in bactericidal assays (Robbins et al., 1965; Bjornson and Michael 1970; Schulkind et al., 1972), although the activity of IgG has been found in rabbit serum to increase during sequential immunisation (Pike and Chandler, 1971). Also, IgG is

predominantly produced in an anamnestic response and is the major antibody class found in the lower respiratory tract of sheep (Gorin *et al.*, 1979). It is doubtful whether IgA can activate bactericidal activity via the classical pathway (Sirotak *et al.*, 1976) and it has even been suggested that IgA may block bactericidal activity against *P. haemolytica* (MacDonald *et al.*, 1983). The immunoglobulin class meriting most attention, following an anamnestic response to vaccination or after the development of pneumonia, would therefore seem to be IgG.

The sera and all but one of the lung washings of "convalescent" lambs were found to exhibit bactericidal activity against *P. haemolytica* A2. In contrast, samples from untreated control animals, which were susceptible to the *P. haemolytica* challenge, had no bactericidal activity. The presence of bactericidal antibodies in the sera and lung washings of the "convalescent" lambs therefore correlated with resistance to *P. haemolytica*. Whether sufficient complement is available in the lung for bactericidal activity to occur is uncertain, but Burrells (1986) has shown that the C3 component of complement increases in the sheep lung during PI3 virus/*P. haemolytica* infection. The possibility that complement behaves as an acute-phase reactant in disease has been suggested previously (Atkinson and Frank, 1980). As the recovery of lung washings involved dilution of the lung fluids, bactericidal activities of sera and lung washings were not directly comparable. Therefore, although the bactericidal capacity of the lung washings was lower than their respective sera, this may not necessarily reflect the situation *in vivo*. The lungs of these "convalescent" lambs may have constituted an environment

as bactericidal for P. haemolytica as their blood. It is perhaps more relevant to note that no bactericidal activity was detectable in the lung washings of control lambs at necropsy.

In attempts to determine which antigen was the target for antibodies involved in the bactericidal assay, absorption with LPS was found to abolish the bactericidal activity of the standard positive serum. Previous authors have described LPS as being the antigen receptor for the bactericidal antibodies directed against other Gram-negative bacteria (Glynn and Ward, 1970; Tramont et al., 1974). An SSE preparation of P. haemolytica A2 also reduced the activity of bactericidal antibodies. This crude extract contained small amounts of LPS, which may have been responsible for the reduction in bactericidal activity, although other cell surface antigens such as outer membrane proteins (Tramont et al., 1974) and phage receptors (Gabay, 1977), have been implicated as targets for bactericidal antibodies. Separation of SSE into its components may therefore reveal additional target antigens.

The bactericidal assay described here will be useful in the understanding of the mechanisms of immunity against P. haemolytica A2 and in monitoring the ability of vaccines to produce bactericidal activity both systemically and in the lung. This assay may have similar applications for the study of bactericidal activity against other Gram-negative bacteria and recent studies with P. multocida (Wijewardana and Sutherland, in press) have indicated this.

Bactericidal activity against the A2 serotype was induced in the sera and lung washings of "convalescent" SPF lambs (Table 4.1) and in SPF lambs vaccinated with a crude homologous preparation of P.

haemolytica cytotoxin (section 8). In contrast to the "rough" type LPS of the serotype A2 , all the T biotype serotypes were found to possess "smooth" type LPS. To investigate further whether antibodies in immune sera would express bactericidal activity to a T biotype organism (serotype T10), sera from animals which were "convalescent" or vaccinated against this serotype were tested for their bactericidal activity. However, these sera were not bactericidal under the assay conditions described here despite the presence in these sera of antibodies to LPS and other cell-surface antigens as demonstrated by an anti-LPS ELISA and immunoblotting respectively.

Together, these results suggest that the T10 serotype of P. haemolytica is, in contrast to the A2 serotype, resistant to the bactericidal effects of antibody and complement. One possible explanation for this distinction is that the LPS from the T10 serotype is of a "smooth" variety whilst the A2 serotype possess a "rough" type of LPS (Fig. 4.3). The LPS of the other T serotypes (3, 4 and 15) were also shown to be "smooth" (Fig. 4.3) therefore also indicating their potential for serum resistance and this would be worthy of further study. However, no immune sera against these serotypes are available at present and this study was therefore not carried out here.

The resistance of T10 serotype to bactericidal activity may indicate why the T serotypes more readily causes a systemic form of ovine pasteurellosis in young adult sheep than the A serotypes, which are generally associated with the pneumonic forms of disease.

Bactericidal activity raised by vaccination has been correlated



with protection against pneumonia caused by the A2 serotype (section 8). The apparent resistance of the T10 serotype to bactericidal activity may therefore have significant bearing on the formulation of vaccines against the systemic form of pasteurellosis caused by the T serotypes.

## SECTION 5

Opsonin-Mediated Phagocytosis of *P. haemolytica* Serotype A2.

It has been noted that *P. haemolytica* serotype A1 is poorly phagocytosed by bovine bronchoalveolar macrophages (BAM) (Benson et al., 1978; Markham and Wilkie, 1980). Opsonising antibodies have, however, been shown to increase phagocytosis (Markham and Wilkie, 1980; Maheswaran et al., 1980). Uptake of late-log phase (18 hour) organisms has also been observed to occur more readily than with early log-phase (6 hour) cultures (Berggren et al., 1981).

A significant increase in the uptake of chromium-51 isotope labelled *P. haemolytica* A2 was shown after opsonisation with sera and lung washings from "convalescent" SPF lambs (Donachie et al., 1986a). However, in agreement with the studies on phagocytosis of *P. haemolytica* A1 by bovine macrophages, the degree of phagocytosis in this study was also low. Despite this, there were indications that opsonisation may be associated with protection of sheep against pasteurellosis, and this mechanism of immunity would therefore seem worthy of further investigation.

Several methods for studying phagocytosis have been developed and these include plate counting of bacteria released from sedimented phagocytes (Watson, 1976), counting visualised internal particles by microscopy (Lee-Smith and Rommel, 1977) and quantitation of isotope-labelled bacteria (Verhoef et al., 1977) including *P. haemolytica* (Maheswaran et al., 1980). Each of these methods has however depended upon the removal of extracellular bacteria, generally involving extensive washing of sedimented

phagocytes by centrifugation, before phagocytosed bacteria can be counted. This practice is tedious, time consuming and can result in cell loss or death which leads to counting errors.

A rapid, microtitre plate, opsonophagocytosis assay was therefore developed to measure the uptake of isotope-labelled P. haemolytica by ovine BAM. The principle of this assay was that non cell-associated bacteria would be removed from assay suspensions by filtration through selectively sized filter membranes (5.0  $\mu\text{m}$  pore size) which would retain BAM and thus allow enumeration of cell-associated (phagocytosed) bacteria. This method was then used to screen sera for their opsonic potential and to characterise opsonophagocytosis of P. haemolytica A2.

Donachie et al. (1986a) used chromium 51-isotope labelled P. haemolytica for opsonophagocytosis assays whereas Maheswaran et al. (1980) employed tritiated thymidine ( $^3\text{H}$ ). The advantages that  $^3\text{H}$  has over chromium-51 is that chromium-51 is spontaneously released from bacteria during the assay period and can be taken up by BAM therefore giving erroneously high background counts, whereas  $^3\text{H}$  is not spontaneously released. Also,  $^3\text{H}$  has a much longer half-life and is therefore more cost-effective in use. Because of these advantages,  $^3\text{H}$  was chosen as the isotope for use in the assay.

$^3\text{H}$ -labelled P. haemolytica A2 was prepared by inoculating 100  $\mu\text{l}$  of P. haemolytica A2 culture, which had been removed from storage at  $-70^{\circ}\text{C}$ , into 10 ml of NB containing 100  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine (Amersham U.K.). The broth was incubated for 18 hours at  $37^{\circ}\text{C}$  and 1ml aliquots were then centrifuged at 11,000 g for 5 min on a microfuge. Pelleted bacteria were washed three times by centrifugation in Hanks' BSS and finally resuspended in Hanks' BSS

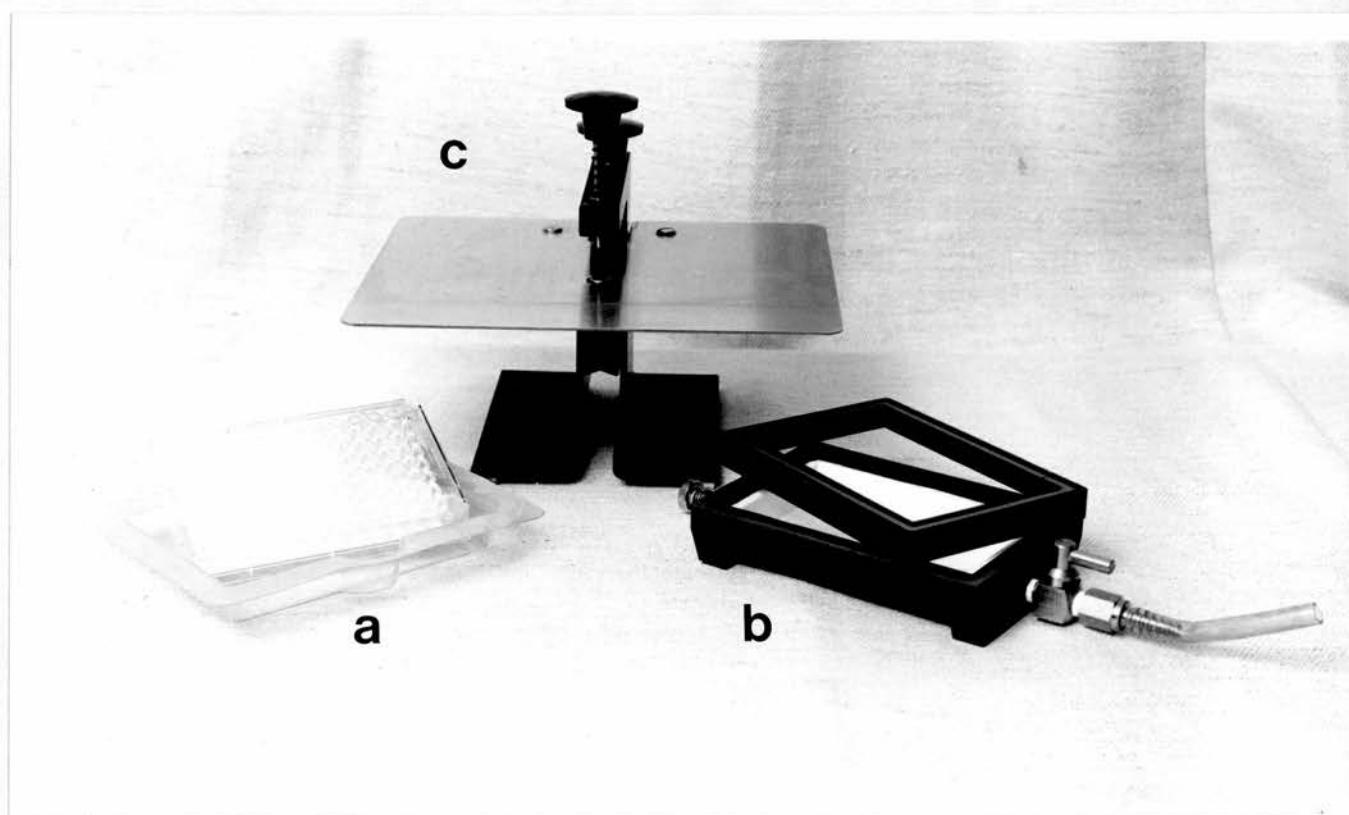
supplemented with 10% heat-inactivated FBS, 2% 30 mM Hepes buffer, 1% sodium bicarbonate and adjusted to pH 7.2 with 1 M Na OH (Hanks' medium) to the desired concentration. Bacterial numbers were determined by retrospective plate counting on SBA plates by the method of Miles et al. (1938).

Sheep BAM were recovered from lungs obtained at Gorgie slaughter house, Edinburgh. Cells were recovered by lung lavage as described by Burrells (1986), followed by sedimentation at 100 g for 20 min at room temperature. Cells were washed twice in Hanks' BSS and resuspended in 5ml of Hanks' medium. An aliquot (10  $\mu$ l) of cells was added to 90  $\mu$ l of trypan blue dye (Sigma, U.K.) and the total cell count and BAM viability assessed by counting in an improved Neubauer cell counting chamber. BAM suspensions were only used if they were > 90% viable and represented greater than 80% of the cell population as assessed by morphological criteria. BAM were finally adjusted to  $2.5 \times 10^6$  viable cells  $\text{ml}^{-1}$  by dilution in Hanks' medium.

Sera to be tested for opsonins (immunoglobulins) were heat-inactivated at  $56^{\circ}\text{C}$  for 30 min before use.

The opsonophagocytosis assay was performed in Millititer-SV microtitre plates (Millipore, Bedford, U.S.A.) using the Titertek vacuum apparatus (Millipore) shown in Fig. 5.1. The microtitre plate filter membranes (5  $\mu\text{m}$  pore size) were pre-wetted by the addition of 10  $\mu$ l of Hanks' medium to each assay well. Bacterial suspension (100  $\mu$ l) was added to each assay well except for three blank control wells which received 100  $\mu$ l of Hanks' medium. Triplicate 100  $\mu$ l volumes of bacterial suspension were also added to 2 ml of Scintillator 299 (Packard Instrument Co. U.S.A.) in

Fig. 5.1. The Titertek microtitre plate apparatus used for opsonophagocytosis assays. a, millititer SV microtitre plate and carrier; b, vacuum apparatus; c, filter punch.





plastic counting vials for counting as total activity controls on a beta-isotope counter (Canberra Packard, U.K.). Volumes (20  $\mu$ l) of test sera, positive control (an SPF lamb "convalescent" serum) and negative control (FBS) sera were then added to triplicate assay wells, the plates sealed with sterile plastic film and incubated at 37°C for 30 min to allow opsonisation to occur. After opsonisation, 80  $\mu$ l of BAM suspension was added to each assay well except the bacteria-only control wells, which received 80  $\mu$ l of Hanks' medium. Plates were again covered with sterile plastic film and incubated at 37°C for the required period to allow phagocytosis to occur.

Extracellular bacteria were removed from assays suspensions by placing assay plates on the Titertek vacuum apparatus, removing assay medium by suction and washing each well five times by suction with 200  $\mu$ l volumes of Hanks' BSS. The retained BAM containing cell-associated bacteria were dried onto membrane filters by placing the assay plates at 37°C in an incubator. Individual well-membranes were then removed with a punch, added to 2 ml of scintillant in plastic vials and activity counted on a beta-isotope counter.

The percentage of bacterial inoculum phagocytosed in each sample triplicate was calculated from the formula :

$$\% \text{ phagocytosis} = 100 \times \frac{(\text{the mean counts per min (CPM) in triplicate test filters} - \text{the mean CPM in bacteria only filters})}{(\text{the mean CPM in the total counts samples} - \text{the CPM in the bacteria only filters})}.$$

It was always confirmed that the CPM in the bacteria only control filters was close to the CPM in the blank control filters

before assay verification, since this ensured that the extracellular bacteria had been removed from assay suspensions by washing.

An opsonic index (OpI) of the percentage of bacteria which were specifically opsonophagocytosed was calculated from the formula:

$$\text{OpI} = 100 \times (\text{CPMs} - \text{CPMc} / \text{CPMt}).$$

Where, CPMs = the mean CPM for phagocytosed bacteria in the sample, CPMc = the mean CPM for bacteria phagocytosed in the presence of the standard negative control serum and CPMt = the mean CPM in the total bacterial inoculum (100  $\mu\text{l}$  volume). All CPM are minus the mean CPM found in the bacteria only control filters.

#### 5.1. Evidence for the retention of BAM on assay filter membranes.

The usefulness of this phagocytosis assay depended upon the assumption that BAM would be retained on the assay plate filters. Evidence for this was sought by adding chromium 51-isotope labelled BAM to assay wells. BAM were labelled exactly as described in section 3, suspended in Hanks' medium at  $2.5 \times 10^6$  viable BAM  $\text{ml}^{-1}$  and 80  $\mu\text{l}$  volumes added to six assay wells containing 120  $\mu\text{l}$  of Hanks' medium. BAM suspension (400  $\mu\text{l}$ ) was also added to six 1 ml polypropylene vials (NUNC, Denmark) containing 600  $\mu\text{l}$  of Hanks' medium which acted as spontaneous release controls. The assay plate and tubes were incubated at  $37^{\circ}\text{C}$  for 30 min to emulate phagocytosis and then the assay plate was placed on the Titertek vacuum apparatus and washed five times in 200  $\mu\text{l}$  of Hanks' BSS. The initial assay medium and each subsequent wash were collected by placing a microtitre plate in the vacuum apparatus below the assay plate. The six spontaneous-release control tubes were centrifuged

at 100 g for 5 min at room temperature to sediment the BAM. The supernatant was removed and the BAM washed five times in 1 ml of Hanks' medium. The supernatants and washings for each sample were combined and added to plastic counting tubes. The BAM pellets from the polypropylene tubes and the dried membranes from the assay plate wells were also added to plastic counting vials and all samples were counted in a gamma-isotope counter (Canberra Packard, U.K.)

The mean CPM in the washings from the microtitre plate assay wells was 2,083 and the mean CPM in the microtitre plate assay filters was 10,003. Therefore 17.2% of the activity was present in the supernatants. The mean CPM in the washings from the spontaneous-release control tubes was 15,095 and the mean CPM in the BAM pellets was 67,274, resulting in a mean spontaneous release of 18.0% in the control tubes. This indicated that the amount of isotope label which passed through the microtitre plate filter membranes was equivalent only to the amount of isotope expected to be spontaneously released. This therefore suggested that BAM were retained by the assay filters, thus validating the assay procedure.

## 5.2 Optimisation of the opsonophagocytosis assay.

Opsonophagocytosis was optimised with respect to 1) bacterial count in ratio to BAM numbers and 2) differing incubation periods. The mean CPM in the *P. haemolytica* A2 suspension (100  $\mu$ l) was 1331, while the blanks and bacteria-only controls gave 111 and 112 mean CPM respectively.

### 5.2.1. Optimisation of *P. haemolytica* A2 to BAM ratio.

BAM at  $2.5 \times 10^6 \text{ ml}^{-1}$  were mixed with *P. haemolytica* A2 in ratios of 1 to 50, 1 to 10, 1 to 5, 1 to 0.5 and 1 to 0.05. The *P. haemolytica* suspension had previously been opsonised, as described, in the presence of either the standard positive serum or the standard negative serum.

Results (table 5.1) showed that maximum phagocytosis occurred when a BAM to bacteria ratio of 1 to 5 was employed. However, the OpI was similar at ratios of 1 to 10 and 1 to 5. At both of these ratios the % phagocytosis was significantly ( $p < 0.001$ ) higher in the opsonised suspensions when compared with the unopsonised ones. At ratios of 1 to 0.5 and 1 to 0.05 the CPM was approximately that of the blanks and no phagocytosis was therefore apparent. At a ratio of 1 to 50 opsonisation did not significantly increase the % phagocytosis. A ratio of between 1 to 5 and 1 to 10 was therefore selected as optimum.

### 5.2.2. Optimisation of assay incubation period.

Opsonophagocytosis in the presence of the standard positive serum was measured at intervals of 10, 20, 30, 40 and 60 mins. The % phagocytosis was 13, 23.1, 22.5, 21.5 and 23.3% respectively for these intervals and the % phagocytosis therefore did not change significantly beyond 20 min incubation. An assay period of 30 mins was selected for further assays.

Table 5.1. The percent phagocytosis of *P. haemolytica* A2 in assays containing differing bacteria to phagocyte ratios

<u>Opsonising serum</u>	<u>BAM: bacteria ratio</u>	Mean ( $\pm$ standard error) <u>% phagocytosis</u>	<u>OpI</u>
standard positive	1:50	7.0 ( $\pm$ 0.18)	4.1
	1:10	24.9 ( $\pm$ 0.2)	19.5
	1:5	32.1 ( $\pm$ 0.4)	20.0
	1:0.5	0	0
	1:0.05	0	0
standard negative	1:50	2.9 ( $\pm$ 0.26)	-
	1:10	5.4 ( $\pm$ 0.3)	-
	1:5	12.1 ( $\pm$ 0.17)	-
	1:0.5	0	-
	1:0.05	0	-

BAM = bronchoalveolar macrophage

% phagocytosis (see text)

OpI - opsonic index (see text)

standard positive serum = serum from a "convalescent" SPF lamb

standard negative serum = foetal bovine serum

### 5.3. Characterisation of the opsonin-mediated phagocytosis of P. haemolytica A2 by BAM.

Both complement components (C3b) and immunoglobulins (IgG subclasses) have been found to increase the uptake of bacteria by phagocytes (Verhoef et al., 1977). The role of complement as an opsonin for P. haemolytica A2 and its interaction with opsonic antibody was therefore investigated. That opsonisation of P. haemolytica by the standard positive serum was due to the presence of immunoglobulins was also confirmed with purified IgG from this serum.

#### 5.3.1. The role of complement as an opsonin for P. haemolytica A2.

Complement (naïve SPF lamb serum stored at  $-70^{\circ}\text{C}$ , see section 4) was added (20  $\mu\text{l}$ ) with either the standard positive serum or the standard negative serum (20  $\mu\text{l}$ ) to phagocytosis suspensions as described above, except that BAM were added as 60  $\mu\text{l}$  volumes of a  $3.3 \times 10^6$  viable BAM  $\text{ml}^{-1}$ . Assay suspensions therefore included 1) 20  $\mu\text{l}$  of complement + 20  $\mu\text{l}$  of standard positive serum, 2) 20  $\mu\text{l}$  of complement + 20  $\mu\text{l}$  of standard negative serum, 3) 20  $\mu\text{l}$  of standard positive serum + 20  $\mu\text{l}$  of standard negative serum and 4) 20  $\mu\text{l}$  of heat-inactivated ( $56^{\circ}\text{C}$  for 30 min) complement + 20  $\mu\text{l}$  of standard negative serum.

Complement with the standard negative serum was found to cause a significant ( $p < 0.05$ ) increase in phagocytosis (13.3%) of P. haemolytica A2 when compared to heat-inactivated complement (4.3%). The standard positive serum induced 22.7% phagocytosis of the bacterial inoculum and this was increased (but not significantly) to 30.0% when complement was added.



### 5.3.2. The opsonic activity of purified IgG for P. haemolytica A2.

Purified IgG ( $2.27 \text{ mg ml}^{-1}$ ) was shown to opsonise P. haemolytica A2 and significantly ( $p < 0.05$ ) increased phagocytosis to 21.2% compared to 4.3% in the absence of antibody.

### 5.3.3. Absorption of opsonising serum with P. haemolytica A2 antigen fractions.

Volumes (1 ml) of the standard positive serum were absorbed with LPS, SSE or crude cytotoxin at  $1 \text{ mg ml}^{-1}$  for 30 min at  $37^{\circ}\text{C}$ . After absorption the serum was sterilised by filtration ( $0.45 \mu\text{m}$  pore sized membrane). The antigens used for absorption were prepared as described in Materials and Methods.

Neither absorption with LPS nor crude cytotoxin significantly altered the opsonic capacity of the standard positive serum, giving 23.3 and 26.4 % phagocytosis respectively. Absorption with SSE, however, caused a small, but significant ( $p < 0.001$ ) decrease in opsonic capacity which resulted in 18.4% phagocytosis compared to 24.9% in the presence of the unabsorbed serum.

## Discussion

The microtitre plate opsonophagocytosis assay was rapid and gave little variation between sample triplicates. Only low (but statistically significant) levels of phagocytosis of P. haemolytica A2 were seen in the presence of opsonins, namely IgG and complement. Low % phagocytosis have been observed previously for P. haemolytica A1 (Benson *et al.*, 1978; Markham and Wilkie, 1980) and the opsonic capacity of the standard positive serum found in this work was similar to levels of phagocytosis found previously

for "convalescent" lamb sera (Donachie et al., 1986a).

The optimal ratio of BAM to P. haemolytica for the demonstration of opsonophagocytosis appeared to be between 1 to 5 and 1 to 10. At a higher ratio (1 to 50) phagocytosis did not increase significantly in the presence of opsonins. Also, it was noted that phagocytosis did not increase significantly beyond a period of 20 min incubation at a BAM to P. haemolytica ratio of 1 to 10. Taken together, these results suggest that phagocytosis of P. haemolytica in vitro is limited. One reason for this may be that BAM are affected by the production of P. haemolytica cytotoxin during the incubation period; and the greater the number of bacteria in the assay the more cytotoxin there would be expected to be produced. Berggren et al. (1981) noted that at higher bacteria to BAM ratios P. haemolytica A1 inhibited phagocytosis by bovine BAM. Furthermore, Sutherland et al., (1983) found that live P. haemolytica A1 organisms were toxic for ovine BAM. The convalescent sera (which was used for opsonisation in this section) contained cytotoxin neutralising (CN) antibodies, but these may not have been sufficient to neutralise cytotoxin, which was presumably continuously produced throughout the assay period. Additionally, any surviving intracellular P. haemolytica A2 may continue to produce small amounts of cytotoxin inside the phagocyte, which would not be neutralised by extracellular CN antibodies.

Despite the low levels of phagocytosis found in vitro, significant increases were demonstrated after opsonisation of P. haemolytica with immune serum. The probable toxic effects of cytotoxin in vitro may also not be so apparent during the early stages of infection in vivo, when only low numbers of P.

haemolytica are likely to be present. At this time the BAM as the resident phagocyte (Reynolds and Newball, 1976) may play an essential role in the clearance of P. haemolytica from the lung. The ability of the immune animal to increase phagocytosis by opsonisation may be a critical mechanism in avoiding establishment of infection. Augmentation of the BAM population by infiltrating PMNL may also contribute to clearance of P. haemolytica from the ovine lung (Davies and Penwarden, 1981). Because a significant effect of opsonisation can be detected in vitro, further characterisation of this mechanism of immunity was considered to be justified.

Both complement (the C3b component) and antibody (particularly the IgG<sub>2</sub> subclass in sheep; Watson, 1976) have been shown to be important opsonins of bacteria (Verhoef et al., 1977). Active serum complement was found in this thesis to induce significant opsonophagocytosis of P. haemolytica A2. Although complement-mediated opsonisation could not be shown to be synergistic with antibody-mediated opsonisation, these opsonins were not, at least, mutually exclusive in that opsonophagocytosis was not impeded in the presence of both antibody and complement. One possible explanation for this lack of synergy is that maximum phagocytosis of P. haemolytica may be around 30% in vitro because of the effects of toxicity already discussed, and therefore significant synergy in opsonisation with antibody plus complement was not apparent because the limit of phagocytosis was nearly reached by antibody alone. Affinity-column purified IgG from the standard positive serum, at a concentration equivalent to that found in the whole serum, was shown to opsonise P. haemolytica A2

at a similar level. This indicated that IgG was the major opsonin in the standard positive serum.

Absorption of bactericidal activity (antibody) from convalescent serum with LPS suggested that this was the target antigen for antibodies involved in this activity (section 4). LPS did not, however, significantly absorb opsonic activity from the standard positive serum when used at  $1 \text{ mg ml}^{-1}$  final concentration. Absorption with crude cytotoxin also failed to decrease phagocytosis, and therefore a role for CN antibodies aiding phagocytosis in vitro was not demonstrated. SSE of P. haemolytica A2 cells did, however, decrease the opsonic capacity of serum. A major antigen in SSE is the serotype specific capsular antigen (Donachie et al., 1984b; Adlam et al., 1985a). Anti-capsular antibodies have been found to opsonise other bacteria such as S. pneumoniae (Giebink et al., 1977) and strains of E. coli (Van Dijk et al., 1979) and it may be speculated, therefore, that the capsular antigen of P. haemolytica A2 is a target antigen for opsonic antibodies.

Recently, monoclonal antibodies (mAbs) against P. haemolytica A1 capsule, and to a lesser degree mAbs to LPS, were shown to be opsonins for mouse macrophages using the method described in this section (C. Wilson, A.D. Sutherland, M. Quirie, L. Inglis and W. Donachie. In preparation.). Production of monoclonal antibodies against P. haemolytica A2 antigens might therefore help to elucidate further the antigens involved in opsonophagocytosis of this serotype.

## SECTION 6

The Susceptibility of In Vivo Grown *P. haemolytica* to Effector Mechanisms of Immunity.

That in vivo grown bacteria can differ phenotypically from bacteria grown in vitro has been noted by others. Differences include the in vivo expression of OMPs involved in iron acquisition (Griffiths et al., 1983; Sciortino et al., 1983; Brown et al., 1984) and changes in LPS composition (Kelly et al., 1989). Bacterial growth in vivo can also lead to phenotypic changes resulting in resistance to bactericidal effects. Sensitive *N. gonorrhoea* grown in guinea pig chambers became resistant to human serum but reverted to the sensitive state on in vitro sub-culture (Penn et al., 1976)

It was considered possible that *P. haemolytica* may also undergo phenotypic change in vivo. As a consequence, changes in relation to expression of virulence factors and interaction with effector mechanisms of immunity may occur. It was therefore decided to investigate the interaction of *P. haemolytica* grown in vivo with bactericidal and opsonophagocytic mechanisms.

Day et al. (1980) studied the production of bacterial products and changes in the surface structure of *S. aureus* after in vivo growth in intraperitoneal chambers. This technique was therefore adopted here for the in vivo growth of *P. haemolytica* A2 in sheep. The resultant organisms were examined for expression of in vivo antigens and for susceptibility to bactericidal and phagocytic immune mechanisms.



### 6.1. Preparation and implantation of peritoneal growth chambers.

Chambers for the growth of *P. haemolytica* A2 *in vivo* were prepared from silicone tubing (19.0 mm internal diameter and 25.4 mm outer diameter) (Altec, Alton. England). Lengths of tubing (approximately 50 mm) were closed at one end with a 25 mm diameter, 0.45  $\mu$ m filter membrane (Millipore, U.K.) which was sealed in place with medical-grade silicone type A adhesive (Dow Corning. U.K.). The chambers were sterilised by autoclaving together with a second filter membrane which was to be used to close the other end of the chamber.

*P. haemolytica* A2 chamber inoculum was prepared by centrifuging bacteria from an 18 hour, 10 ml NB culture at 1,000 g for 30 min and washing the pellet once in phosphate buffered saline pH 7.2 (PBS). The washed bacteria were resuspended in 50 ml of PBS, and approximately 10 ml volumes were pipetted aseptically into the chambers. With the aid of sterile forceps, the chambers were sealed with the other sterile filter membrane. Retrospective plate counting of the bacterial inoculum resulted in counts of  $2.0 \times 10^7$  and  $5 \times 10^6$  cfu ml<sup>-1</sup> on two separate occasions.

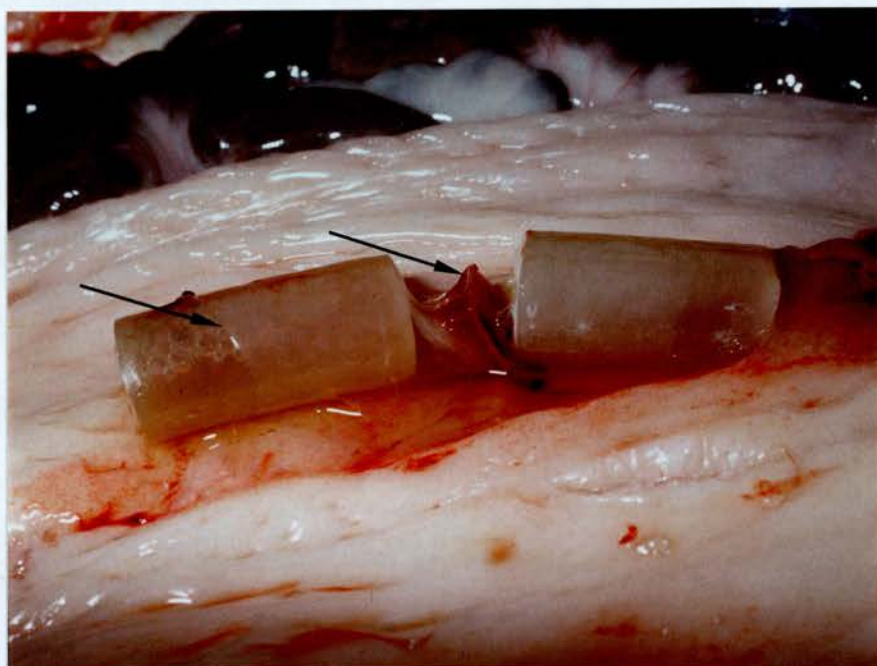
Two chambers were each implanted aseptically into the peritoneal cavity of anaesthetised sheep by Miss Lorna Hay and Dr. Gareth Jones. The peritoneal opening was closed with surgical suture and the animals allowed to recover. Seven days later the animals were killed and the chambers recovered.

At necropsy the chambers were found to be completely enclosed by a thick fibrinous capsule (Fig.6.1) from which they were removed aseptically with a scalpel. Considerable areas of ecchymoses were



Fig. 6.1. Necropsy photograph of chamber-implants in the peritoneum of a sheep. A fibrinous capsule from which the chambers were removed is arrowed.

Fig. 6.1. Necropsy photograph of chamber-implants in the peritoneum of a sheep. A fibrinous capsule from which the chambers were removed is arrowed.



noted on the peritoneal lining surrounding the chambers. The chamber contents were recovered by standing the chambers on one end and cutting away the upper filter with sterile scissors. The contents of individual chambers were pooled on each occasion. No white blood cells were found on microscopic examination of the chamber contents, and this was taken as an indication that the chambers had not ruptured in vivo. However, a small fibrinous clot was found in each chamber which was removed with sterile forceps. A plate count of the bacteria in the pooled chamber-contents showed that  $3.45 \times 10^7$  and  $2.0 \times 10^7$  cfu ml<sup>-1</sup> P. haemolytica A2 were present in pure culture on two separate occasions.

Bacteria were recovered from volumes (10 ml) of the chamber fluid by centrifugation at 1,000 g for 30 min at 4°C and the supernatant fluid was stored at -70°C until required. Aliquots of chamber contents (including bacteria) were also stored without washing at -70°C for later analysis. Bacteria pelleted by centrifugation were washed once in PBS and on resuspension in the required diluent (see below) were tested for susceptibility to bactericidal activity and phagocytosis without passage by growth in vitro. Also, some pelleted bacteria were lyophilised and stored for later analysis.

#### 6.2. The susceptibility of in vivo grown P. haemolytica A2 to phagocytosis.

The susceptibility of in vivo grown organisms to phagocytosis by BAM after opsonisation with "convalescent" SPF lamb serum could not be assessed by the method described in section 5 since this

required incorporation of  $^3\text{H}$ -thymidine into bacteria by growth in vitro. Opsonophagocytosis was therefore assessed by a plate counting method and in vitro grown bacteria were tested in the same manner for direct comparison.

In vivo grown bacteria were resuspended in Hanks' medium after pelleting from peritoneal-chamber contents by centrifugation. In vitro grown bacteria were prepared from an 18 hour culture in the same manner as described in section 5, except  $^3\text{H}$  thymidine was omitted from the culture. Bacterial counts in both inocula were confirmed by retrospective plate counting.

The BAM suspension was prepared as described in section 5 and sera used as opsonins were the standard positive and negative sera described in section 5.

The opsonophagocytosis assay was performed on two separate occasions. Volumes (500  $\mu\text{l}$ ) of in vivo or in vitro grown P. haemolytica A2 were mixed with 100  $\mu\text{l}$  of the standard positive and negative sera in duplicate 1 ml polypropylene vials (NUNC, Denmark). Tubes were incubated at  $37^\circ\text{C}$  for 30 min on a roller apparatus (Luckham Laboratories) to allow opsonisation to occur. Then, 400  $\mu\text{l}$  of BAM suspension was added to each suspension and the tubes further incubated for 30 min on the roller apparatus to allow phagocytosis to occur. After phagocytosis, BAM were sedimented by centrifugation at 100 g for 5 min at  $4^\circ\text{C}$ , washed three times to remove extracellular bacteria, resuspended in 1 ml of 0.1% Triton X100 (Sigma) and finally vortexed vigorously to lyse BAM and release intracellular bacteria. The lysed BAM suspensions were then titrated in ten-fold dilutions in peptone water (Gibco) and each dilution plated for retrospective bacterial counting.



The mean percentage of P. haemolytica A2 phagocytosed in duplicate sample tubes was calculated from the formula:

mean % phagocytosis =  $100 \times (\text{The mean cfu ml}^{-1} \text{ in lysed BAM suspensions} / 0.5 \times \text{the mean cfu ml}^{-1} \text{ in the assay inoculum})$ .

An opsonic index (OpI) was calculated for each suspension using the formula:

$$100 \times (C_s - C_c / C_t).$$

Where  $C_s$  = the mean counts from phagocytosed bacteria in sample suspensions opsonised with the standard positive serum,  $C_c$  = the mean counts from phagocytosed bacteria in sample suspensions opsonised with the standard negative serum and  $C_t$  =  $0.5 \times$  the mean counts from the total number of bacteria in the assay inoculum.

The P. haemolytica inocula gave counts of  $2.9 \times 10^7$  and  $1.0 \times 10^7$  cfu ml<sup>-1</sup> of assay suspension for in vivo grown organisms and  $3.9 \times 10^7$  and  $1.0 \times 10^7$  cfu ml<sup>-1</sup> of assay suspension for in vitro grown organisms respectively on the two separate assay occasions.

Results (Table 6.1) showed that BAM phagocytosed a mean of 21.8% and 21.5% of the in vitro grown bacterial inoculum on the two separate occasions in the presence of the standard positive serum while a mean of 0.002% and 0.7% of the in vitro grown bacterial inoculum was phagocytosed in the presence of the standard negative serum. The levels of opsonophagocytosis of the in vitro grown bacteria in the presence of the standard positive serum were similar to those seen in the microtitre-plate phagocytosis assay (section 5). The mean % phagocytosis of in vivo grown bacteria was also increased in the presence of the standard positive serum (9.65% and 8.1%) when compared to the standard negative serum (1.9% and 4.7%) on the two separate assay occasions, but this was a



Table 6.1. The mean percentage phagocytosis of P. haemolytica A2 grown in vivo or in vitro.

<u>Assay</u>	<u>Bacterial growth conditions</u>	<u>Opsonising serum</u>	<u>% phagocytosis</u>	<u>OpI</u>
1	<u>in vivo</u>	standard positive	9.65	7.8
		standard negative	1.9	0
	<u>in vitro</u>	standard positive	21.8	21.8
		standard negative	0.002	0
<hr/>				
2	<u>in vivo</u>	standard positive	8.1	3.4
		standard negative	4.7	0
	<u>in vitro</u>	standard positive	21.5	20.8
		standard negative	0.7	0

Standard positive serum = a "convalescent" immune specific-pathogen-free lamb serum.

Standard negative serum = a naive specific-pathogen-free lamb serum

lower percentage of opsonophagocytosis than was determined for in vitro grown bacteria and resulted in a mean OpI of 5.6% over the two assays compared with a mean OpI of 21.3% for in vitro grown bacteria tested under the same assay conditions.

These results indicated that in vivo grown P. haemolytica A2 organisms were less susceptible to antibody-mediated opsonophagocytosis than in vitro grown organisms and suggested that phenotypic changes occur in organisms grown in vivo which reduce their susceptibility to opsonophagocytosis.

#### 6.3. The susceptibility of in vivo grown P. haemolytica A2 to bactericidal activity.

In vivo grown bacteria were tested on two separate occasions for their susceptibility to antibody-mediated complement-dependent bacterial killing by the method described in section 4. In vitro grown organisms were tested in the same assay and considered to be known susceptible organisms (section 4). The standard positive and negative sera and the assay method were as described in section 4.

The inoculum for in vivo grown organisms was prepared by resuspending organisms in modified barbitol buffer (MBB) and bacterial counts in the assay inocula were found by retrospective plate counting to be  $1.7 \times 10^4$  and  $1.1 \times 10^4$  cfu ml<sup>-1</sup> on the two separate assay occasions.

The in vitro grown bacteria were from 3 hour cultures and were prepared as described in section 4. Bacterial counts in the assay inoculum were found by retrospective plate counting to be  $1.0 \times 10^4$  and  $1.8 \times 10^4$  cfu ml<sup>-1</sup> on the two separate assay occasions.

Results (Table 6.2) showed that the in vitro bacterial inocula were killed by a mean of 100% and 87% in the presence of the standard positive serum and 0% by the standard negative serum on the two separate assay occasions. These results indicated that the assay procedure was functional and that in vitro bacteria required the addition of antibody and complement for bacterial killing to occur. This confirmed the findings of section 4. In contrast, in vivo grown bacteria were killed by a mean of 97% and 100% in the presence of the standard positive serum and 91% and 90% in the presence of the standard negative serum.

These results led to suggestions that either phenotypic changes in in vivo grown organisms had induced susceptibility of these organisms to the alternative complement pathway or that in vivo grown bacteria had cell-surface bound host-derived immunoglobulin. An examination was therefore made for the presence of cell-surface bound immunoglobulin.

#### 6.4. Examination of in vivo grown P. haemolytica A2 for bound, host-derived immunoglobulin.

Host derived immunoglobulin which was bound to in vivo grown P. haemolytica A2 was detected by direct interaction of the immunoglobulin with donkey anti sheep-immunoglobulin conjugated with horse radish peroxidase.

##### 6.4.1. Detection of immunoglobulin by dot immunoblotting.

An aliquot (1 ml) of in vivo grown P. haemolytica A2 cells was recovered from  $-70^{\circ}\text{C}$  storage, thawed, pelleted on a microfuge, washed twice in 1ml of MBB and resuspended in 1 ml of MBB. A 2  $\mu\text{l}$

Table 6.2. The mean percentage killing (%K) of in vitro and in vivo grown P. haemolytica A2 in the bactericidal assay

<u>Assay</u>	<u>Bacterial growth conditions</u>	<u>Serum</u>	<u>Mean % K</u>
1	<u>in vivo</u>	standard positive	97
		standard negative	91
	<u>in vitro</u>	standard positive	100
		standard negative	0
<hr/>			
2	<u>in vivo</u>	standard positive	100
		standard negative	90
	<u>in vitro</u>	standard positive	87
		standard negative	0

Standard positive serum = a "convalescent" immune specific-pathogen-free lamb serum.

Standard negative serum = a naive specific-pathogen-free lamb serum

volume of this suspension was adsorbed onto a nitrocellulose strip (Millipore, U.K.) and air dried. An 18 hour in vitro culture of P. haemolytica was treated in a similar manner and acted as a negative control for the presence of bound sheep immunoglobulin. A 2  $\mu$ l volume of the standard positive serum was also adsorbed onto nitrocellulose to act as an IgG positive control. Dot immunoblotting was carried out as described in Materials and Methods.

The in vivo grown P. haemolytica A2 organisms were found to have bound sheep immunoglobulin detectable by dot immunoblotting whereas the in vitro grown organisms did not (Fig.6.2). Immunoglobulin was also detectable in the positive control.

#### 6.4.2. Detection of bound IgG by western blotting.

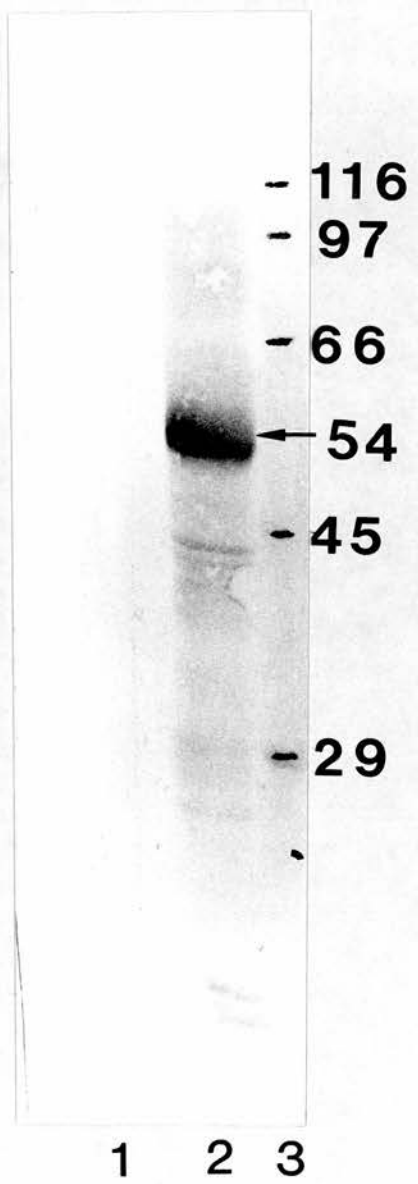
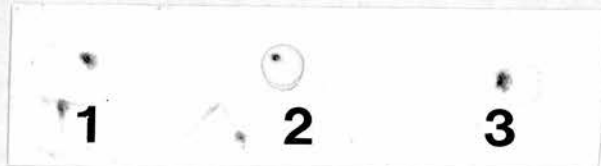
Immunoglobulin bound to in vivo grown P. haemolytica A2 was further characterised by western blotting as described in Materials and Methods. Lyophilised P. haemolytica A2 organisms (300  $\mu$ g), which were from either peritoneal chambers or 18 hour NB cultures grown in vitro, were resuspended in SDS-PAGE solubilising buffer and run on 10% gels. Resolved antigens were transferred to nitrocellulose and reacted with donkey anti-sheep immunoglobulin conjugated with HRP.

A thick band of 53 kDa was detected by the conjugate on the track containing in vivo grown organisms. The in vitro grown organisms did not contain this band (Fig. 6.3). By its molecular mass and its reaction with the conjugate, the band was identified as the heavy chain of sheep IgG which was previously identified in Materials and Methods (part 2.7).

Fig. 6.2. Dot immunoblot of samples of *P. haemolytica* A2 whole cells grown: (1), in vitro; (2), in vivo; and (3), a "convalescent" immune specific-pathogen-free lamb serum containing IgG. Samples were reacted with donkey anti-sheep immunoglobulin conjugated with horseradish peroxidase to detect sheep immunoglobulin.

Fig. 6.3. Western blot of *P. haemolytica* A2 whole cells grown: lane 1, in vitro, lane 2, in vivo and lane 3, molecular mass markers. Samples were reacted with donkey anti-sheep immunoglobulin conjugated with horseradish peroxidase to detect sheep immunoglobulin.





6.5. SDS-PAGE analysis of in vivo grown P. haemolytica A2 organisms.

Donachie and Gilmour (1988) have recently reported that P. haemolytica A2 organisms isolated from the pleuritic fluid of infected animals expressed envelope-located proteins which were either not detectable, or present in detectably lesser amounts, in bacteria grown in NB in vitro. Some of these proteins (particularly 70 and 100 kDa) could be expressed in vitro when grown in iron depleted media and were therefore considered to be IRPs. SDS-PAGE analysis to detect these IRPs was used to confirm that the bacteria recovered from peritoneal chambers had undergone phenotypic change during in vivo growth. Kelly et al. (1989) have recently found that the LPS recovered from in vivo grown P. aeruginosa organisms had additional polysaccharide bands which they suggested may enhance the virulence of this organism in vivo. In vivo grown P. haemolytica A2 organisms were therefore examined for their LPS phenotype by SDS-PAGE.

6.5.1. Examination of proteins from in vivo grown P. haemolytica A2 by SDS-PAGE.

SDS-PAGE for the detection of proteins was carried out as described in Materials and Methods. Lyophilised whole bacterial cells (300 µg) from either peritoneal chambers or in vitro grown 18 hour NB cultures were resuspended in solubilising buffer and loaded onto 10% SDS-PAGE gels. Gels were stained with Coomassie blue dye (Morrissey, 1981) to visualise protein bands.

Figure 6.4 shows that protein bands which were additionally produced or detectably over-produced were identified in in vivo

grown cells when compared to in vitro grown cells. These bands were 105, 100, 95, 70, 66, 53 and 25 kDa in molecular mass. Furthermore, protein bands of 36 and 24 kDa were expressed by in vitro grown organisms which were not detectable in in vivo grown organisms.

#### 6.5.2. Examination of LPS from in vivo grown P. haemolytica A2 by SDS-PAGE.

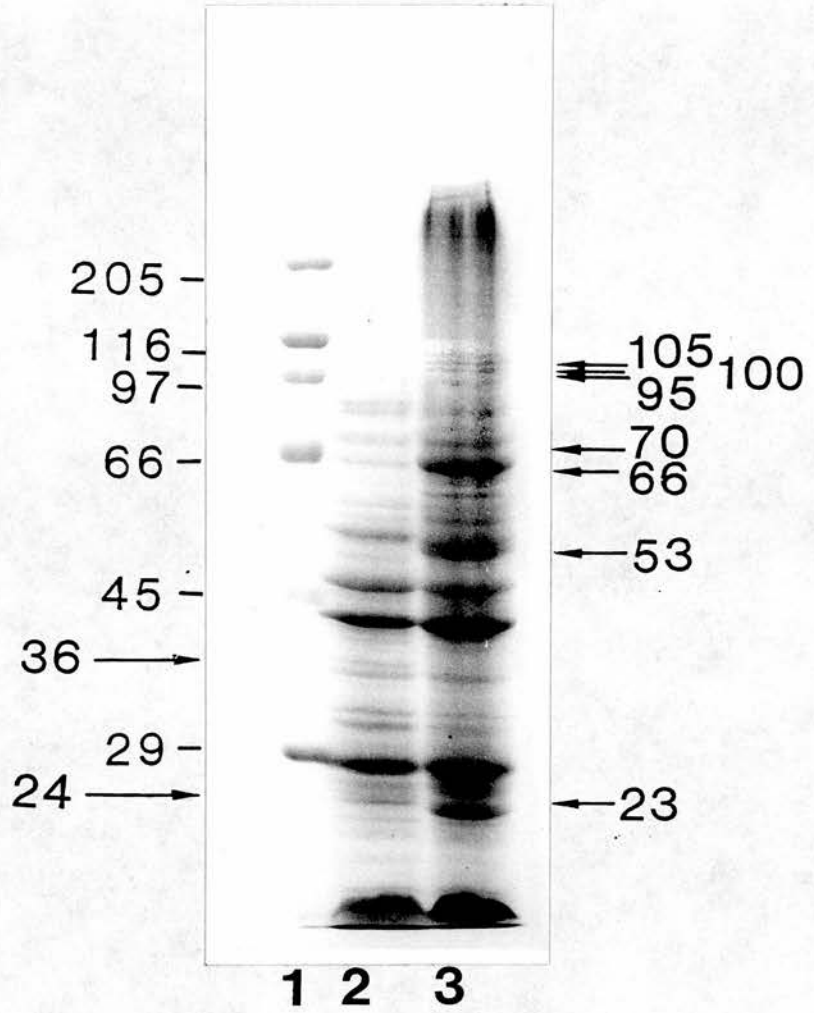
LPS from both in vivo and in vitro grown cells was examined by SDS-PAGE of proteinase K digested whole bacterial cells as described in Materials and Methods. Lyophilised cells (300 µg) were resuspended in solubilising buffer and proteinase K and loaded onto 12.5% gels.

Both in vivo and in vitro grown cells were found to possess rough type LPS (results not shown) which gave an identical profile to the serotype A2 LPS shown in section 4 (Fig. 4.3). No phenotypic change in the A2 LPS structure was therefore detectable after in vivo growth.

#### 6.6. Detection of a cell-wall capsule on in vivo grown P. haemolytica A2.

The production of a capsule in S. aureus has been associated with avoidance of opsonophagocytosis by prevention of opsonin phagocyte-receptor interaction (Wilkinson et al., 1979). Early log-phase cultures of P. haemolytica A1 have been shown to produce more capsule than late log-phase cultures (Corstvet et al., 1982) and it is reported that early log-phase P. haemolytica A1 are less susceptible to opsonophagocytosis than late log-phase cultures. The

Fig. 6.4. SDS-PAGE of: lane 1, molecular mass standards,  
lane 2, in vitro grown and lane 3, in vivo  
grown P. haemolytica A2 whole cells.



size of the P. haemolytica capsule might therefore be implicated as a factor in avoidance of phagocytosis. Since in vivo grown P. haemolytica A2 organisms were found to be opsonophagocytosed to a lesser degree than similarly treated in vitro grown organisms, in vivo grown organisms were compared to early log-phase and late log-phase in vitro grown organisms for capsule content.

Detection of capsule was carried out retrospectively after the findings of phagocytosis studies. In vivo grown organisms were therefore examined for capsule either after storage at  $-70^{\circ}\text{C}$  or upon recovery of organisms from a third chamber implant. Capsule was detected by the Maneval stain (Maneval, 1941). In vitro grown P. haemolytica A2 were recovered from 10 ml volumes of NB grown for 6, 18 or 24 hours. Bacteria were harvested from 1 ml volumes by centrifugation at 11,000 g for 5 min and pelleted bacteria resuspended in 1 ml of PBS pH 7.2. Similarly, in vivo grown bacteria were pelleted from chamber fluid by centrifugation followed by resuspension in 100  $\mu\text{l}$  of PBS pH 7.2. A loopful of each bacterial suspension was mixed on a microscope slide with a loopful of a 1.0% aqueous solution of Congo red stain (BDH chemicals.), spread thinly and then air dried. The smears were then counter-stained with a 1.0% aqueous solution of acid fuchsin suspended in Maneval solution A (30 ml of 5% aqueous phenol, 8 ml of 20% aqueous glacial acetic acid and 4 ml of 30% aqueous ferric chloride) for 2 min, drained and blotted dry. Negatively stained capsules were seen by this method to surround red staining bacterial bodies presented on a pale blue background.

P. haemolytica A2 organisms from 6 hour in vitro cultures (Fig. 6.5.1) were found to have thicker capsules when compared to



bacteria from 18 and 24 hour cultures (Fig. 6.5.2 and 6.5.3 respectively). Both, In vivo grown organisms recovered from  $-70^{\circ}\text{C}$  storage (Fig 6.5.4), and in vivo grown organisms recovered directly from peritoneal chambers (not shown) did not appear to have any more capsule than was seen on organisms from 18 hour cultures. Since 18 hour in vitro grown organisms were used for comparison with in vivo grown organisms it therefore appeared unlikely that capsular content was the reason for the differences in opsonophagocytosis observed between these type of organisms. In vivo grown organisms were, however, found to be aggregated into clusters of bacteria (Fig. 6.5.4). This aggregation may have inhibited the optimal binding and distribution of opsonic antibodies and/or may have constituted too large a particle for optimal uptake by phagocytes. Either of these possibilities could have given rise to the decreased levels of phagocytosis observed with in vivo grown bacteria.

Fig. 6.5.1. Maneval stain showing the capsule surrounding  
P. haemolytica A2 organisms grown for 6 hours  
in vitro.

Magnification x 2,000

Fig. 6.5.2. Maneval stain showing the capsule surrounding  
P. haemolytica A2 organisms grown for 18 hours  
in vitro.

Magnification x 2,000

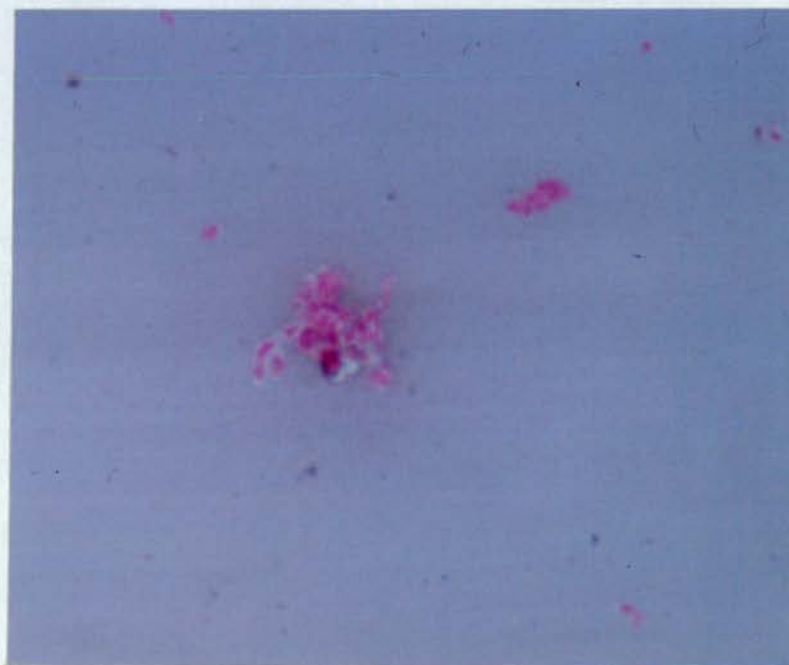


Fig. 6.5.3. Maneval stain showing the capsule surrounding  
P. haemolytica A2 organisms grown for 24  
hours in vitro.

Magnification x 2,000

Fig. 6.5.4. Maneval stain showing the capsule surrounding  
P. haemolytica A2 organisms grown in vivo.

Magnification x 2,000





## Discussion.

The method of Day et al. (1980) was used successfully to grow pure cultures of P. haemolytica A2 organisms in vivo. The bacteria were viable, in relatively large numbers and free of contaminant host cells.

Day et al. (1980) found that the formation of a fibrinous capsule around peritoneal implant chambers was associated with virulent strains of S. aureus and was probably due to chemotaxis, since the fibrinous capsule was composed mainly of PMNL.

Implanted chambers containing P. haemolytica A2 were also found in this thesis to promote the formation of a capsule around the chambers. This may suggest that chemotactic factors are also produced by P. haemolytica A2. Areas of ecchymoses were also noted on the peritoneum adjacent to the chambers and may have suggested the production of soluble toxin(s) such as proteases by bacteria in the chambers.

In vivo grown organisms were found to be as susceptible to antibody-mediated complement-dependent bacterial killing as were in vitro grown organisms. In vivo grown organisms were also found, however, to be killed in the absence of added specific antibodies. The detection of surface adherent (host-derived) antibodies on in vivo organisms (part 6.4) suggested that this was responsible for the effect, rather than phenotypic changes causing susceptibility of in vivo grown P. haemolytica A2 to killing by the alternative complement pathway. The finding that in vivo grown organisms had no detectable structural changes in LPS antigens, when compared with in vitro grown organisms supported this conclusion, since LPS was detected as a target antigen for antibodies involved in



bactericidal mechanisms (section 4). The presence of host-derived antibodies may also have been responsible for the slightly elevated levels of phagocytosis observed for in vivo grown organisms in the presence of the standard negative serum when compared to similar suspensions of in vitro grown organisms. The binding of IgG to S. epidermidis grown in peritoneal dialysate has been observed by others (Williams et al., 1988).

On two separate occasions, in vivo grown organisms were found to be opsonophagocytosed to a lesser degree than similarly treated in vitro organisms (part 6.2). In vitro and in vivo grown organisms were therefore compared for the presence of capsule to determine if over-production of this factor in vivo may be responsible for the reduced susceptibility to opsonophagocytosis. In vivo grown organisms were however found to possess similar amounts of capsule when compared to in vivo grown organism from 18 hour cultures, which were employed for phagocytosis studies. Since large quantities of capsule were not detected in in vivo grown organisms this was not a reason for the apparent reduction in susceptibility of these organisms to phagocytosis. In vivo grown organism were, however found in aggregates and these may have afforded these organisms some resistance to phagocytosis.

Early log-phase P. haemolytica A2 (6 hour) cultures were found to possess larger amounts of capsule than organisms from 18 or 24 hour cultures. This finding is in agreement with that of Corstvet et al. (1982), who reported similar findings for capsular production in P. haemolytica A1.

Donachie and Gilmour (1988) detected the in vivo expression of outer-membrane proteins from organisms recovered from the pleuritic

fluid of experimentally infected animals. Similar expression of these antigens in organisms recovered from peritoneal chambers was therefore taken as evidence of phenotypic change in the bacterial chamber inoculum in response to the in vivo environment. Organisms from chambers were found to express increased amounts of 100 and 70 kDa antigens. These antigens were detected by Donachie and Gilmour (1988) from in vivo derived organisms and were further proven to be IRPs which were inducible in vitro by growth in iron depleted medium. In this thesis, two further proteins were detected as being produced by in vivo grown organisms. These proteins were of 95 and 105 kDa in molecular mass. It is interesting to note that recombinant cytotoxin has been found to consist either of a 102 kDa antigen (Lo et al., 1986) or of a 105 kDa antigen which results in a 95 kDa degradation product in older cultures (Chang et al 1987). These novel bands in in vivo organisms could therefore be evidence of increased levels of intracellular cytotoxin. Also, proteins of 53, 23 and 66 kDa were found in in vivo cells. The 53 kDa protein may have been heavy chain IgG since bound IgG was detected on in vivo cells as a 53 kDa band (part 6.4.2.) and purified IgG was found to consist of a 53 kDa heavy chain and a 26 kDa light chain in section 2. The 66 kDa protein may have been sheep albumin which was bound to in vivo cells.

Attempts to detect the production of cytotoxin by in vivo grown organisms were not made since substantial indirect evidence of toxin production in vivo has already been reported. This evidence consists of reports of CN antibodies being raised in cattle which have recovered from either natural or experimental infection with P. haemolytica (Baluyut et al., 1981; Shewen and Wilkie, 1983a;

Cho et al., 1984; Gentry et al., 1985a), and in this thesis "convalescent" lambs have been shown to produce CN antibodies after infection (section 3).

Two proteins (36 kDa and 24 kDa) were found to be produced by in vitro organisms which were not expressed by in vivo organisms. These proteins may be useful in distinguishing between animals which have been vaccinated with in vitro derived products and those which have undergone natural infection.

The findings that in vivo grown organisms are susceptible to antibody-mediated bactericidal mechanisms, and to some extent opsonophagocytosis, lends further support to the suggestion that these mechanisms may play an important role in immunity and that the in vitro assays for detecting these mechanisms may therefore help in analysing the protective potential of vaccines.

## SECTION 7

Protection of Lambs by Passive Transfer of Immune Serum.

Wells et al. (1979) failed to protect lambs by passive protection with serum from lambs that had been vaccinated against P. haemolytica A1. This led these authors to conclude that humoral immunity alone was incapable of protecting against experimental pasteurellosis. Serum from "convalescent" lambs however was later found to passively protective in a mouse infection model (Donachie et al., 1986b) and in this thesis humoral effector mechanisms of immunity such as cytotoxin neutralisation by antibody (section 3), antibody-dependent bacterial killing (section 4) and opsonophagocytosis (section 5) have been associated with immunity in "convalescent" SPF lambs.

A re-evaluation of the protection afforded by the transfer of immune serum to SPF lambs challenged with P. haemolytica therefore seemed merited. It was considered that the demonstration of immunity via passive transfer of immune serum might prove the importance of humoral immunity. Furthermore, examination of the sera by in vitro assays may further confirm the role of these mechanisms of immunity in protection and support the value of these in vitro tests as correlates of immunity.

#### 7.1. Production of immune sera for passive transfer experiments.

Immune sera were produced in conventionally reared Cheviot lambs from the same flock located at New Milton farm, which is part of

the

Moredun Research Institute research facilities. Sera were obtained either from "convalescent" or vaccinated lambs.

#### 7.1.1. "Convalescent" lambs.

Chronic ("atypical") pneumonia was reproduced experimentally at different times in two groups of lambs by minor variations of the general methodology described previously (Jones *et al.*, 1986). Homogenised lung lesion suspensions made from naturally occurring cases of "atypical" pneumonia (LH) were injected intratracheally (i.t.) in 8 to 10 ml volumes on day 0. Two different suspensions were used. The first (injected into group Cv1) was treated with 2 mg ml<sup>-1</sup> of ampicillin (Penbritin, Beecham Animal Health) and contained Mycoplasma ovipneumoniae as the only detectable microorganism, present at  $5 \times 10^6$  colour changing units (ccu) ml<sup>-1</sup>. The second, which was not treated with antibiotic, was injected into group Cv2 and contained M. ovipneumoniae ( $5 \times 10^6$  ccu ml<sup>-1</sup>), Mycoplasma arginini ( $5 \times 10^5$  ccu ml<sup>-1</sup>) and P. haemolytica A2 ( $1 \times 10^{3.9}$  cfu ml<sup>-1</sup>).

Group Cv1 (6 lambs) was injected intramuscularly (i.m.) with 5 mg kg<sup>-1</sup> liveweight of ampicillin at the time of LH inoculation. The same lambs were injected again on days 7, 50, 56 and 93 with 2 ml i.t. and 2 ml intranasally (i.n.) of 5 hour NB cultures of P. haemolytica A2 diluted appropriately in PBS. These inocula contained  $1 \times 10^{4.5}$ ,  $1 \times 10^{4.5}$ ,  $1 \times 10^{6.3}$  and  $1 \times 10^{8.0}$  cfu ml<sup>-1</sup>, respectively.

Group Cv2 (6 lambs) was inoculated in like manner on days 4 and 40 with inocula containing  $1 \times 10^{7.2}$  and  $1 \times 10^{5.6}$  cfu ml<sup>-1</sup>, respectively, of P. haemolytica A2.

#### 7.1.2. Vaccinated lambs.

Individual animals were injected subcutaneously three times, on each occasion with 2 ml of vaccine prepared by adsorption of antigen with aluminium hydroxide gel (Alhydrogel, Miles Research Products) and emulsification with Bayol and Arlacel A. Group V1 (contemporary with Group Cv1) was injected with a mixture of heat-killed organisms (HKO, 1 mg ml<sup>-1</sup>) and SSE (2.5 mg ml<sup>-1</sup>) of P. haemolytica A2 (Gilmour et al., 1983). Each dose was administered 28 days apart. Group V2 (contemporary with Group Cv2) was injected with a vaccine containing SSE only (2.5 mg ml<sup>-1</sup>) given 21 and 22 days apart.

#### 7.1.3. Collection of serum and formation of immune serum pools.

Blood in 200 to 300 ml volumes was taken on four occasions between Weeks 9 and 14, and the animals were exsanguinated in Weeks 14 or 15. Serum pools were formed from all bleedings of the three or four animals in each group that were serologically negative before the experiment.

#### 7.1.4. Production of control serum

Two batches of control sera were used for experiment 1. One (CtS1) was made up of sera taken from 3 to 6 week-old (w.o.) SPF lambs; the other (CtS2) was made up of sera taken over 2 weeks from 20 to 25 conventionally reared lambs aged 4 to 6 weeks.



For experiment 2, a serum pool (CtS3) was formed from four peers of those used to produce CvS2 and VS2. These donors were serologically negative when first screened, but low transient titres to *P. haemolytica* were detected by anti-SSE ELISA in three of them during later bleeds.

#### 7.1.5. Preparation of semi-purified immunoglobulin-rich fractions.

For experiment 1, immunoglobulin-rich fractions (IRF) were prepared from 3 l volumes of CvS1, VS1 and CtS2 by cold ethanol extraction (Cohn *et al.*, 1946; Oncley *et al.*, 1949). These fractions (CvIg1, VIg1 and CtIg2, respectively) were resuspended in 200 ml normal saline solution (0.85%) and sterilised by passing through filters of 0.22  $\mu\text{m}$  average pore diameter.

IRF for subsequent experiments were prepared by precipitation with 40% (v/v) saturated ammonium sulphate (Garvey *et al.*, 1977) from 1.8 l volumes of CvS2 and VS2 for Experiment 3 (CvIg2 and VIg2i, respectively) and from 1.9 l of VS2 for Experiment 4 (VIg2ii). The precipitates were resuspended in normal saline and dialysed exhaustively against normal saline until free of  $\text{SO}_4^{2-}$  ions as determined by the addition of 10% barium chloride (Garvey *et al.*, 1977). After filtration through 0.45  $\mu\text{m}$  membranes, the suspensions were concentrated using either polyethylene glycol (Breox 20M PEG, Chemical Services and Distribution, Crewe) or lyophilisation followed by resuspension in a minimum volume of distilled water. The suspensions were further dialysed against normal saline, adjusted with saline to a volume considered

appropriate for the experiment and sterilised by filtration. Total volumes produced were 250 ml each of CvIg2 and VIg2i, and 380 ml of VIg2ii.

## 7.2. Characterisation of serum pools and IRF (Table 7.1).

IgG was estimated turbidimetrically (Seneviratne and Moores, 1980) and IgM nephelometrically by an adaptation of the method of Buffone *et al.* (1975) using an IL Multistat III microcentrifugal analyser (Instrumentation Laboratory U.K.). Albumin was determined by the bromocresol-green dye binding method of Doumas *et al.* (1971).

Antibodies to *P. haemolytica* A2 were assayed by indirect haemagglutination (IHA) (Fraser *et al.*, 1983), ELISA using SSE (Donachie and Jones, 1982) or lipopolysaccharide (LPS) bound to assay plates by the method of Fodor and Donachie (1988), cytotoxin neutralizing (CN) assay (Section 3), bactericidal assay (Section 4) and opsonophagocytosis assay (section 5).

IgG:albumin ratios were 0.56 to 1.20 in whole serum (Table 7.1). They were increased 9.9 to 11.0 fold by cold ethanol extraction and 6.7 to 8.2 fold by ammonium sulphate precipitation. IgM:albumin ratios were increased 8.5 to 8.7 fold by cold ethanol extraction and 3.0 to 4.8 fold by ammonium sulphate precipitation.

All sera and IRF pools from vaccinated or convalescent animals were positive and those from control animals negative by anti-SSE ELISA; IRF generally demonstrated higher titres than their parent serum pools. All pools tested by anti-LPS ELISA were positive, including CtS3 which had a low titre. The CN assay produced very similar findings, except that VS1 was negative. The bactericidal

Table 7.1. Characterisation of serum pools and immunoglobulin-rich fractions

Experiment	Group	Inoculum	Content (g/l)			Titre <sup>a</sup>				Bact assay (%)	opsonophagocytosis assay (OpI)
			IgG	IgM	Albumin	IHA	SSE	ELISA	CN		
								LPS			
1	1	CvS1	26.0	0.7	27.1	16	1172	6500	200	100	40.8
		+ CVIg1	30.3	0.7	3.2	2	5434	3111	800	100	1.3
	2	VS1	16.7	0.7	35.6	0	2597	12091	<100	86	7.7
2	1	+ VIg1	29.1	0.6	3.5	2	9060	10292	100	100	0.6
		CtS1+2	17.6	0.2	31.7	2	0	NA	<100	0	17.4
	3	+ CtIg2	40.5	1.2	4.1	0	0	NA	<100	36	1.07
3	1	CvS2	27.1	0.7	30.2	32	1046	3183	200	91	38.6
		CtS3	31.2	0.7	31.1	2	0	2615	<100	0	15.1
	2	CvIg2	150.2	1.5	21.8	128	2748	NA	1600	0	32.4
4	1	VIg2(i)	221.3	1.6	22.4	32	62502	24369	1600	0	27.0
		VS2	32.0	0.4	26.7	8	9060	6697	100	90	21.1
	3	VIg2(ii)	147.9	1.0	18.5	32	41996	8342	400	88	15.4

<sup>a</sup>Mean of duplicate tests using *P. haemolytica* A2 antigens. IHA = indirect haemagglutination test; ELISA = enzyme-linked immunosorbent assay; CN = cytotoxin neutralisation assay; Bact = bactericidal; OpI = opsonic index; SSE = sodium salicylate extract; LPS = lipopolysaccharide; %K = percent killed; NA = not available; Cv = convalescent; V = post-vaccinal; Ct = controls; S = serum; Ig = immunoglobulin-rich fraction

assay distinguished immune from control serum and IRF, although some bactericidal activity was detected in CtIg2 and none was detected in CvIg2 or VIg2(i). The IHA test distinguished immune from control serum, except in experiment 1, where three immune products yielded insignificant titres. The opsonophagocytosis assay gave similar results to the IHA test, and the three sera which gave low IHA titres in experiment 1 also gave low OpI. In general, the highest titres in the IHA test were produced by convalescent sera and IRF.

### 7.3. Passive protection experiments

The design of these experiments is shown in Table 7.2.

Infective inocula: Pl3 virus, produced as described previously (Sharp et al., 1978), was injected i.t. and i.n. in 8 ml and 2 ml volumes respectively. Two preparations of virus inoculum were used which contained  $1 \times 10^{6.25}$  and  $1 \times 10^{6.17}$  of a 50% tissue culture infective dose (TCID<sub>50</sub>) per 0.2 ml.

P. haemolytica was administered by aerosol as described elsewhere (Gilmour et al., 1975). Titres of P. haemolytica from particles <3.3  $\mu$ m in diameter, as indicated by the growth from the bottom stage of a 3-stage Porton impinger (May, 1966), ranged from  $1 \times 10^{6.98}$  to  $1 \times 10^{8.43}$  cfu ml<sup>-1</sup>.

Virus, when used, was given 7 days before P. haemolytica. Day 0 was the day of first exposure to either agent.

Large volumes (60 to 430 ml) of serum were administered 24 h before P. haemolytica infection by intraperitoneal (i.p.) injection into the right sublumbar fossa using a flutter valve. Small volumes of IRF (18 to 59 ml) or serum (30 ml, Group 3,

Table 7.2. Design of passive protection experiments

Experiment	Lambs at day 0 Age (days)	Weight (kg)	Challenge method	Treatment of Groups			Immunising dose (ml/kg)	
				1	2	3	4	i.p.(S) i.v.(Ig)
1	67-69	12.5 - 19.5	PI3+PhA2	CvS1+ CvIg1 <sup>a</sup> (614)	VS1+ VIg1 (628)	CtS1+CtS2 +CtIg2 (444)	-	22 1.4
2	25-26	5.7 - 13.9	PI3+PhA2	CvS2 (542)	CtS3 (624)	-	-	20 -
3	20-21	5.9-11.2	PI3+PhA2	CvIg2 (451)	VIg2i (664)	VS2 (365)	None	11.4 (S i.v. and i.p.) <sup>b</sup>
4	30-31	5.2-14.1	PhA2 only	VIg2ii (606)	None	-	-	4.1

Cv = Convalescent; V = post-vaccinal; Ct = Control; S = Serum; Ig = immunoglobulin-rich fraction; i.p. = intraperitoneal injection given 24h before challenge with P. haemolytica; i.v. = intravenous injection given 3-4h before P. haemolytica;

PI3 - parainfluenza 3 virus; PhA2 - P. haemolytica A2.

<sup>a</sup>Figures in parentheses indicate total IgG administered as mg per kg liveweight

<sup>b</sup>Groups 1 and 2; <sup>c</sup>Group 3 was given 11.4 ml serum per kg, 30 ml i.v. and the remainder i.p.

Experiment 3) were given by intravenous (iv.) injection 3 to 4 hours before exposure to aerosol. In Experiment 4, where i.v. doses were particularly large, the animals were pre-sedated with Saffan (Glaxovet). Serum or IRF was warmed to 37°C before injection.

#### 7.4. Clinical, microbiological, histopathological and serological examinations and necropsy

A disease index was employed, based on four components, in which the maximum score per lamb was 60.

(i) Clinical score. Animals were examined from Days 1 to 6 inclusive and necropsied on Day 7. One point was allotted for each observation of raised rectal temperature ( $>40.5^{\circ}\text{C}$ ), dyspnoea or coughing, severe depression, and death or euthanasia on humanitarian grounds. The maximum daily score was 4 and the experiment score 24. Animals which died prematurely were scored 4 for each day remaining in the experiment.

(ii) Pneumonia score. The mean area of consolidated lung tissue on dorsal and ventral surfaces was assessed by computer-aided measurement from lung diagram charts completed at necropsy. This area, expressed as a percentage of the total lung area, was halved to give the pneumonia score (maximum 22). Animals which died rapidly, without development of consolidated lung tissue, were scored maximum if isolations of P. haemolytica indicated lung infection and a generalised septicaemia (one or more of heart blood, spleen and liver positive).

(iii) P. haemolytica isolation index. Lung tissue samples from eight different sites were combined to give four pools as follows:



right apical and cardiac lobes; left apical and cardiac lobes; right diaphragmatic and intermediate lobes; and either side of the left diaphragmatic lobe. These samples were titrated and cultured, and the counts of viable P. haemolytica estimated. The mean log titre of the four pools was taken as the isolation index figure (maximum 8).

(iv) Pleurisy. Fibrinous pleuritic adhesions were scored 1 for each lobe affected, the apical and cardiac lobes of each side being regarded as one lobe for this purpose (maximum 4). Gelatinous fibro-cellular pleurisy with excess pleuritic fluid was scored 3 for each side affected (maximum 6).

Surviving lambs were killed at Day 7 by barbiturate overdosage. Lung tissue pools were examined for bacteria and viruses as described previously (Sharp et al., 1978), and representative lung blocks were fixed in 10% formol saline for histopathological examination. Sera were examined for antibodies to P. haemolytica.

#### 7.5. Experimental results of passive protection experiments.

Experiments 1 and 2: virtually no evidence of pneumonia was detected in the 19 animals treated with immune material and only one (from experiment 2) yielded P. haemolytica from the lungs, in low numbers (Table 7.3). In contrast, three of the six control lambs in experiment 1 died rapidly and four had consolidated lesions in the lungs.

All six control lambs in experiment 2 yielded P. haemolytica from the lungs ( $P < 0.01$  compared with Group 1), but marked evidence of pneumonia was apparent in only two, of which one died and one

had extensive lung consolidation.

Experiment 3: three lambs died of PI3 virus infection on Days 5 and 6. The remainder were then randomised on pre-experiment weights into three groups of seven and one ((Group 4) of eight lambs for challenge with P. haemolytica.

No deaths occurred in any of the treated lambs and evidence of disease in them was minor (Table 7.3). Mean isolation titres were less than  $1 \times 10^2$  cfu g<sup>-1</sup> in all six of the 21 animals treated with immune serum which yielded P. haemolytica from the lungs. In contrast, seven control lambs died, all with evidence of septicaemia. The sole survivor yielded a mean titre of only  $1 \times 10^{2.4}$  cfu P. haemolytica g<sup>-1</sup> of lung tissue, but had consolidation involving 94% of the lung surface area.

Experiment 4: clinical signs of disease were virtually absent in the group treated with immune Ig, although three lambs showed 5-8% consolidation of the lung surface area; two of these yielded low numbers of P. haemolytica (Table 7.3). All but one control animal died of septicaemia, most on Day 3. P. haemolytica was isolated from a shoulder joint, but not the lungs of the sole survivor.

Histopathologically, the majority of animals in experiments 3 and 4 showed interstitial pneumonia with or without a non-suppurative exudate. A small proportion, represented in every group, showed mild to moderate neutrophil presence with or without lung consolidation. Changes typical of pasteurellosis were found in only one animal of Group 3, experiment 3. Fibro-cellular pleurisy was observed only in untreated animals, with four positive in both experiments 3 and 4.

Table 7.3. Clinical and necropsy findings in Experiments 1, 2, 3 and 4

Experiment	Group	N	Source of treatment materials	No. dying or killed in extremis	Mean clinical score (24) <sup>a</sup>	Mean pneumonia score (22) <sup>a</sup>	No. with P. haemolytica pleurisy	No. with P. haemolytica in lungs	Group mean disease index	%P
1	1	6	Convalescent (S + Ig)	0	0	0	0	0	0	100
	2	6	Vaccinate (S + Ig)	0	1.0	0.2	0	0	1.2	95
	3	6	Control (S + Ig)	3	9.5	9.5	2	4	24.7	0
2	1	7	Convalescent (S)	0	0.3	0	0	1**	0.7**	94
	2	6	Control (S)	2	2.7	5.7	1	6	12.7	0
3	1	7	Convalescent (Ig)	0**	0.9**	0.3**	0*	1**	1.3**	97
	2	7	Vaccinate (Ig)	0**	0.3**	0.3**	0*	3	1.1**	97
	3	7	Vaccinate (S)	0**	0.4**	0.6**	1	2*	1.1**	97
	4	8	None	7	11.8	22	5	8	43.1	0
4	1	8	Vaccinate (Ig)	0***	0.4***	1.4**	2*	2*	2.5**	95
	2	9	None	8	16.8	19.6	7	8	47.0	0

CS = Combined score (maximum = 60). Otherwise abbreviations as in Table 7.1.

$$\%P = \% \text{ Protection} = 100 \times \left[ 1 - \left( \frac{\text{disease index of test}}{\text{disease index of control}} \right) \right]$$

<sup>a</sup>Figures in parentheses indicate maximum possible score

\* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001, all with respect to the untreated control group within the relevant experiment according to the Mann-Whitney two-tailed T-test.

## Discussion

These studies have demonstrated that serum or IRF administered i.v. or i.p. can provide virtually total protection against experimental pasteurellosis. Protection was produced by two separate batches of convalescent serum, by post-vaccinal serum produced with two forms of vaccine and by IRF produced from these convalescent and post-vaccinal sera. These findings thus support other work indicating that protection against respiratory disease can be mediated by humoral elements alone (Lam and Switzer, 1971; Masiga et al., 1975), in contrast to the conclusion of Wells et al. (1979) that humoral immunity per se is incapable of preventing pneumonia, specifically ovine pasteurellosis.

Gilmour et al. (1983) found that Serotype A2 SSE/HK0 vaccines similar to those used in this study gave only 40 to 50% protection in SPF lambs, whereas Donachie et al. (1986a) observed that SPF lambs convalescent from experimental infection with P. haemolytica A2 were completely resistant to subsequent challenge with the homologous serotype. However, in this study similar levels of passive protection (between 94 and 100%) were afforded by serum both from vaccinated and convalescent animals. This anomaly may have been due to several reasons: (i) Excessive dosages of serum and IRF may have obscured differences in levels of protective antibodies raised by these two protocols. Generally, convalescent serum and IRF pools had higher bactericidal assay (%K), CN and IHA titres than did vaccinate serum and IRF pools. (ii) A considerable boost in antibody titre was invariably seen in these studies by anti-SSE ELISA following the third vaccination. Gilmour et al. (1983) used only a double-vaccination schedule. (iii) Prior

exposure of donor animals to P. haemolytica may have supplemented the smaller range of antibodies stimulated by the SSE vaccine, thereby raising the protective efficacy of post-vaccinal serum to equivalence with the convalescent serum. Pre-screening indicated that donor animals were negative for antibodies to P. haemolytica A2 by the anti-SSE ELISA, but some of their peers were positive by this test and two yielded P. haemolytica from nasal swabs. In experiment 2 also, control serum from untreated peers of immune serum donors appeared to offer some protection (Table 7.3) and antibodies to LPS were present at low titre (Table 7.1). This indicates that the anti-SSE ELISA lacked the ability and/or sensitivity to detect low levels of protective antibodies.

IRF preparations were as effective as their parent serum pools in these studies. Being no more than semi-purified, the precise nature of their protective components cannot be deduced, although compared with serum their proportional contents of IgG and IgM were increased at the expense of albumin and, presumably, other serum components. Since dosages used were equilibrated by IgG content, this suggests that immunoglobulin was responsible for the protection engendered.

The use of a P. haemolytica-only infection in experiment 4 was designed to ascertain whether prior infection with PI3 virus was responsible, wholly or in part, for the protective effects of injected materials seen previously. PI3 virus can cause epithelial damage and oedema (Rushton et al., 1979), effects which could have promoted the exudation and accumulation in the lungs of blood constituents, including the injected materials. The 95% protection observed in Group 1, experiment 4, indicates



that viral pre-infection was not essential for efficacy of treatment and that either normal immunoglobulin transudation into the bronchoalveolar tree was sufficient or that protection was provided mainly or exclusively intravascularly.

Death after acute or hyperacute septicaemia occurred in some control animals of all experiments, but the 3 to 4.5 w.o. lambs of experiments 3 and 4 had virtually no pulmonary phagocytic cell response, compared with the extensive neutrophil and macrophage exudate present in the lungs of three 9.5 w.o. control lambs of experiment 1. This difference suggests that phagocytic cell function and responses in the young lamb are deficient and mature only after 5 weeks of age. Naturally-occurring field cases of pasteurellosis in lambs 2 months old are generally of septicaemic form (Gilmour, 1978). In the absence of severe respiratory disablement, the cause of death in such cases is presumably endotoxaemia. The ability of immune serum and IRF to protect the immunologically immature lambs of experiments 3 and 4 suggests that protection was independent of phagocytes, i.e. non-opsonic. Instead, the injected immunoglobulins may have been bactericidal, prevented bacterial adherence, enhanced clearance by the muco-ciliary blanket and/or neutralized bacterial metabolic function or products, such as endotoxin or cytotoxin.

The immune sera used for passive protection experiments varied in their abilities to stimulate activity in in vitro assays measuring bactericidal activity, CN and opsonophagocytosis. These sera were however invariably capable of inducing passive protection against P. haemolytica A2. This perhaps indicates the multifactorial nature of protective immunity. Further demonstration



of whether any of these functional mechanisms of immunity can alone provide adequate protection would (if it were possible) require the use of vaccines containing single antigens which stimulate the individual immune mechanisms.

## SECTION 8

Protection of Lambs by Vaccination with a Crude Cytotoxin Preparation

Serotype A2 was the least immunogenic of P. haemolytica serotypes when SSE vaccines were evaluated against homologous challenge in lambs, and significant protection was obtained only when heat-killed organisms were included (Gilmour et al., 1983). However, Donachie et al. (1986a) subsequently showed that "convalescent" lambs were completely protected against a further homologous A2 challenge. This suggested that effective immunisation against the A2 serotype was possible and that the antigens involved in protection were either absent from or present in insufficient amounts in the SSE vaccines tested.

Isolates of P. haemolytica produce an extracellular cytotoxin which is lethal for ovine leucocytes (Sutherland et al., 1983; Sutherland, 1985) and which may be an important pathogenic determinant in ovine pneumonic pasteurellosis. This extracellular toxin was unlikely to have been a component of SSE vaccines which failed to adequately protect against P. haemolytica A2 (Gilmour et al., 1983). It was considered, therefore, that a toxoid might provide protection against P. haemolytica A2, although, toxin-neutralising activity in itself may not be sufficient. Vaccines based on cholera toxoid were found to be more protective when additionally including somatic antigens which stimulated the clearance of organisms (Svennerholm and Holmgren, 1976). Therefore, two vaccines were prepared, one of which contained a crude form of cytotoxin, the other the crude cytotoxin plus SSE. These

toxin-based vaccines were compared with an SSE vaccine similar to that described previously (Gilmour *et al.*, 1983).

Serum antibody responses were measured by an enzyme-linked immunosorbent assay (ELISA) for antibodies to SSE cell surface antigens (Donachie *et al.*, 1986a), an IHA test to measure anti-capsular antibodies (Donachie and Jones, 1982), a cytotoxin-neutralisation (CN) test (section 3), a bactericidal test (section 4) and an opsonophagocytosis assay (section 5). Results from these tests were compared for correlation with protection against *P. haemolytica* A2 infection, and significance testing between grouped serological data was carried out using the Mann Whitney test for non-parametric data.

#### 8.1. Production of a cytotoxin preparation

Crude cytotoxin was prepared as described in Materials and Methods. *P. haemolytica* A2 cultures in dialysis sacs submerged in 500 ml volumes of nutrient broth were incubated for 24 h at 37°C. Sac contents were centrifuged at 10,000 g for 20 min to pellet bacteria and the culture supernatant fluid, containing cytotoxin, was filter-sterilised and lyophilised after exhaustive dialysis against distilled water.

The toxicity end-point (section 3) of the crude cytotoxin used for vaccination was at a concentration of 2.5 mg ml<sup>-1</sup>.

#### 8.2. Production of SSE

The SSE was prepared as described in Materials and Methods with

the additional step that bacterial cell pellets were washed once in PBS to minimise contamination of the extracts with extracellular cytotoxin.

### 8.3. Formulation of vaccines

Three vaccines, which consisted of 0.27% Alhydrogel adjuvant (Miles Laboratories) adsorbed to lyophilised antigen resuspended in distilled water and were formulated as follows: (i) 10 mg ml<sup>-1</sup> of crude cytotoxin; (ii) 2.5 mg ml<sup>-1</sup> of SSE; (iii) 10 mg ml<sup>-1</sup> of crude cytotoxin plus 2.5 mg ml<sup>-1</sup> of SSE.

### 8.4. Experimental procedure.

Vaccine studies were carried out on lambs which were caesarian-derived, colostrum-deprived and maintained in isolation. They were fed sterilised cows' milk, hay and concentrates, and designated specific pathogen-free (SPF) lambs.

#### 8.4.1. Experimental infections

The experimental infection of lambs with P. haemolytica A2 was preceded by infection with PI<sup>3</sup> virus, a method which consistently induces severe pneumonia in SPF lambs (Sharp et al., 1978). The virus was administered intratracheally (8 ml) and intranasally (2 ml) at a titre of  $1 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>. Infection with P. haemolytica A2 was achieved by exposure for 15 min to an aerosol of strain X205A at approximately  $4 \times 10^7$  colony forming units l<sup>-1</sup> and was administered 7 days after infection with PI3 virus.

#### 8.4.2. Experimental design

Three-week-old SPF lambs were allocated randomly into four groups. The crude cytotoxin vaccine group and the control group were allotted eight animals. Groups treated with the SSE and cytotoxin plus SSE vaccines were allotted 14 animals each to ensure that sufficient animals were available for statistical comparison between these vaccine groups. One animal in each of the cytotoxin, SSE and control groups died prior to experimental infection with PI3 virus. Three groups were vaccinated on Days 0 and 28 with 1 ml doses of each vaccine, while one group remained unvaccinated. All animals were given PI3 virus on Day 35 and an aerosol of P. haemolytica A2 on Day 42. Lambs were bled for serum on Day 42. Clinical examinations (section 7) were carried out for 6 days after P. haemolytica infection and surviving animals were necropsied on Day 49.

#### 8.5. Clinical and pathological findings.

Clinical, microbiological and pathological assessments of the response of each lamb were made according to the methods described in section 7.

A high degree of protection was afforded to animals vaccinated with crude cytotoxin both with and without the addition of SSE, while SSE-vaccinated animals were protected significantly less ( $P < 0.01$ ) than the cytotoxin plus SSE group (Table 8.1).

Table 8.1. Group mean experimental disease indices and percentage protection.

Vaccine group	N	Deaths	Disease index <sup>a</sup>	% Protection <sup>b</sup>
Cytotoxin	7	1	7	86
SSE	13	6	26	47
Cytotoxin/SSE	14	0	1	98
Unvaccinated	7	7	50	0

<sup>a</sup>Disease index - the sum of the clinical, consolidated lesion, P. haemolytica isolation index and pleurisy scores (section 7, this thesis)

$$^b \% \text{ Protection} = \left( 1 - \frac{\left[ \text{disease index of treated group} \right]}{\left[ \text{disease index of unvaccinated group} \right]} \right) \times 100$$



#### 8.6. Serological analysis.

All vaccinated animals had serum antibodies to P. haemolytica when measured by SSE-ELISA, while unvaccinated animals had none (Table 8.2). None of the animals had IHA titres indicating that none of the vaccines had induced an antibody response to capsular antigens. The mean CN titres and bactericidal capacities of sera from the cytotoxin and cytotoxin/SSE vaccine groups were significantly ( $P < 0.001$ ) greater than those of the SSE vaccine and unvaccinated control groups. Only two animals of the SSE vaccine group had serum CN activity, both of low titre, indicating that the SSE antigen contained negligible amounts of cytotoxin. None of the vaccinated or untreated control animals had serum OpI  $> 1.0$ . Antibodies involved in opsonophagocytosis were therefore not stimulated by any vaccine.

Serum CN titres and bactericidal capacities of experimental lambs correlated inversely ( $P < 0.001$ ) with the severity of disease measured by an index taking account of clinical, pathological and microbiological parameters (section 7).

Table 8.2. Assay findings for post-vaccinal sera as measured by SSE-ELISA, CN, and bactericidal assay

Vaccine Group	Group mean antibody titre ( $\pm$ standard error) in:		Group mean bactericidal activity ( $\pm$ standard error) %K
	SSE-ELISA	CN Assay	
Cytotoxin	3819 ( $\pm 2704$ )	15.1 ( $\pm 4.3$ )	100 ( $\pm 0.3$ )
SSE	7171 ( $\pm 1166$ )	0.1 ( $\pm 0.1$ )	77 ( $\pm 4.0$ )
Cytotoxin/ SSE	5360 ( $\pm 957$ )	38.7 ( $\pm 10.6$ )	96 ( $\pm 2.5$ )
Unvaccinated	0	0.0	38 ( $\pm 5.8$ )

## Discussion

In this study, a vaccine containing crude cytotoxin plus SSE was 98% protective against experimental infection of lambs with P. haemolytica A2 and the protection afforded by the cytotoxin alone (86%) was only slightly (not significantly) less. However, the protection (47%) given by the SSE vaccine was significantly ( $P < 0.01$ ) poorer and similar to values found previously (Gilmour et al., 1983). Serological analysis revealed no significant difference in the mean %K of groups given crude cytotoxin or crude cytotoxin plus SSE vaccines, whereas the SSE vaccine group had a significantly lower mean %K. The antigens in the crude cytotoxin thus stimulated bactericidal activity more effectively than SSE. Similar ELISA titres in all groups showed there was no significant difference in levels of antibodies to SSE cell surface antigens. Therefore, it was concluded that SSE was not an additional requirement for protective cytotoxin-based vaccines. This is in contrast to vaccines based on Cholera toxoid which show improved protection with the addition of somatic antigens (Svennerholm and Holmgren, 1976). With the crude cytotoxin vaccines used in this thesis, bactericidal activity was probably induced by contaminant lipopolysaccharide (LPS), since antibodies involved in the bactericidal activity of immune serum can be absorbed by the addition of LPS (section 4). The presence of LPS in crude cytotoxin has been detected by SDS-PAGE and a sensitive *Limulus* amoebocyte lysate assay (section 3).

Since the partially protective SSE vaccine produced negligible CN titres and low bactericidal activity, either CN antibodies or high bactericidal capacity, or both, are probably required for

significant protection. Both these variables were significant correlates of immunity ( $P < 0.001$ ) and were stimulated by the crude cytotoxin vaccine.

Whether protection can be ascribed to either CN or bactericidal antibodies alone can only be determined if different antigens are responsible for inducing these antibodies and separation and identification of these can be achieved. However, the most efficient vaccine formulation for *P. haemolytica* may include those antigens that induce both CN and bactericidal antibodies as found in the crude cytotoxin preparation. In addition, inclusion of antigens that stimulate opsonising antibodies for phagocytosis (if these can be found and presented in the correct manner) may be beneficial for protection since this mechanism of immunity correlated in animals convalescent to *P. haemolytica* A2 (Donachie *et al.*, 1986a; section 5, this thesis)

None of the vaccines used in this section of the thesis induced opsonophagocytic antibodies or IHA titres in SPF lambs. The IHA test detects capsular specific antibodies. If the capsule of *P. haemolytica* A2 is a target for opsonins as it is for other bacteria (Giebink *et al.*, 1977; Van Dijk *et al.*, 1979; Penaredondo *et al.*, 1988), then the lack of specific anticapsular antibodies may explain why these vaccines did not induce opsonophagocytic antibodies.

## SECTION 9

General Discussion.

The aim of this thesis was to examine those humoral effector mechanisms involved in immunity to P. haemolytica A2 infection. This involved the development of in vitro assays, for investigating the influence which effector mechanisms had on P. haemolytica. Studies included the use of "convalescent" sera to characterise these assays and correlate them with immunity. Characterisation also involved the detection of P. haemolytica antigens which stimulated specific antibodies involved in the immune mechanisms described. These methods allowed antigen preparations to be selected for the production of a novel vaccine. This vaccine was found to be capable of stimulating protective immunity against experimental P. haemolytica A2 infection.

From the outset, the cytotoxin of P. haemolytica was considered to be potentially of prime importance as a protective antigen, assuming that its toxic activity could be neutralised by specific antibodies. The supposed importance of the toxin stemmed from the evidence given of its lethal effects on ovine leucocytes (Sutherland et al., 1983; Sutherland, 1985; Sutherland and Donachie, 1985), which gave rise to the suggestion that the cytotoxin was a major virulence factor in ovine pneumonic pasteurellosis. Cytotoxin antigens had not previously been included in vaccines for P. haemolytica and, as such, represented possible novel protective antigens for inclusion in experimental vaccines. The development of a protective vaccine against the A2 serotype of P. haemolytica was of particular importance since attempts to

protect against this serotype had been unsuccessful with a previous experimental vaccine formulation. This was despite the fact that this vaccine had proven protection against other *P. haemolytica* serotypes (Gilmour *et al.*, 1979; Gilmour *et al.*, 1983). Further evidence was therefore sought that the cytotoxin was an important virulence factor of *P. haemolytica* A2 and that cytotoxin neutralising (CN) antibodies might be involved in protection.

A crude cytotoxin preparation from the A2 serotype was produced in the same manner as, and compared with, that produced by the A1 serotype, as previously described by Sutherland and Redmond (1986). The crude cytotoxin from the A2 serotype produced potent levels of toxic activity which were detectable by a chromium-51 release assay. It was found, however, that the A2 crude cytotoxin was noticeably less toxic than the A1 toxin preparation. Also, comparison of SDS-PAGE profiles showed lesser amounts of 95 and 105 kDa antigens in the A2 crude cytotoxin compared to those in the A1 preparation. These antigens have recently been identified as cytotoxin antigens by Chang *et al.* (1987). These findings therefore suggested that the A2 serotype produced lesser amounts of extracellular cytotoxin than the A1 serotype. This observation is in agreement with that of Gentry *et al.* (1988), who also showed that the extracellular A2 cytotoxin was present in less detectable amounts than toxin from other serotypes. These results were of interest when compared with previous reports in which Sutherland and Donachie (1985) showed that live *P. haemolytica* A2 organisms were as toxic for sheep bronchoalveolar macrophage (BAM) as were A1 organisms. Combined, these findings might suggest that serotype A2 either has larger amounts of intracellular cytotoxin than the A1



serotype, or that the A1 serotype multiplies faster than the A2 serotype, therefore producing more toxin in the growth medium. Neither of these possibilities was examined in this thesis and studies on this aspect would perhaps benefit from the availability of monoclonal antibodies against the cytotoxin.

In considering the extracellular production of cytotoxin by P. haemolytica further, it is worthwhile comparing the export of proteins by other Gram-negative bacteria. It has been debated whether proteins, including exotoxins, can be truly secreted from the cytoplasm across both inner and outer membranes into the medium. Rather, it seems that most proteins may be exported from the cytoplasm into the periplasmic space and released from there on cell lysis or by secretion in outer membrane vesicles (Schwartz, 1984). Colicins, for example, appear to be released after cell-lysis which is produced by a lysis protein (Mock and Schwartz, 1978; Jakes and Model, 1979). E. coli heat-labile toxin (LT) is mostly located in the cytoplasm, and the smaller amounts found in the medium are mainly associated with outer membrane vesicles (Gankema et al., 1980). It is interesting to note, however, that cholera toxin, which is antigenically cross-reactive with, and chemically similar to, LT is found in abundance in the medium (Germanier et al., 1976). This, at least, indicates that two similar toxins can be secreted in different manners by two different species of bacteria. The outer membrane vesicles with which LT is associated may also be functional in the mode of action of the toxin, being implicated in the interaction of LT with target cell membranes (Gankema et al., 1980; Middeldorp and Witholt, 1981).

Examination of crude cytotoxin preparations by SDS-PAGE showed that both LPS and outer membrane proteins were released into the culture medium. Added to this, previous work by Chang et al. (1984) has shown that toxic activity is susceptible to periodate and amylase treatment, suggesting a role for carbohydrate moieties in the cytotoxin. Also, Sutherland and Redmond (1986) found that the native form of the active toxin was very large in size, as determined by its elution profile from gel-filtration columns. The toxic fraction was also shown to contain LPS and outer membrane proteins. With this evidence it could therefore be speculated that P. haemolytica cytotoxin is secreted in association with membrane vesicles. Differences in extracellular cytotoxic activity between the A1 and A2 serotypes of P. haemolytica might therefore be associated with differences in mechanisms of secretion between these two serotypes.

In possible contrast to this suggestion, however, are the findings of Lo et al. (1986), who showed a large degree of homology between P. haemolytica A1 cytotoxin and E. coli alpha haemolysin. Alpha haemolysin is an actively secreted protein (Springer and Goebel, 1980) which has cistron-encoded components involved in export. This finding might suggest that the A1 cytotoxin is actively secreted from the cell and not released in outer membrane blebs. Recent work A. Lainson (personal communication) indicates that differences in sequence analysis and restriction enzyme digestion-profiles exist between A1 and A2 cytotoxin genes. It would therefore be of interest to determine if these differences are associated with mechanisms of secretion and the different levels of extracellular activity observed between the two serotypes.

Besides outer membrane proteins, LPS and capsular antigens being identified in crude cytotoxin preparations by SDS-PAGE, proteases were also identified in these preparations. The A1 serotype was found to produce a large (Rf 0.05) protease on SDS-PAGE gels incorporating casein (section 3). The size and activity of this protease was unaffected by heating in the presence of mercaptoethanol, suggesting that the size, conformation and activity of the protease were not dependant upon disulphide bridges. The A2 serotype produced an extracellular protease which was much smaller (Rf 0.99) than that produced by the A1 serotype and sensitive to heat treatment with mercaptoethanol (section 3). Otulakowski et al. (1983) speculated that protease activity detected in cytotoxin preparations may be involved in toxin activity. If this were so, the differences in the proteases produced by the A1 and A2 serotypes could also be associated with the lower levels of activity found in the A2 cytotoxin preparations. Proteases have been found to be involved in the activation of toxins produced by other species. Clostridium botulinum type A toxin and E. coli LT are both produced as protoxins which require cleavage by a protease for activation (Thorne and Gorbach, 1981).

The proteases could also be virulence factors. Some proteases, such as those found in N. meningitidis, N. gonorrhoea and E. coli, have been found to split IgA and may help these organisms to evade mucosal immunity (Plaut et al., 1975). Inclusion of P. haemolytica proteases in vaccines may therefore be beneficial in protection if they are antigenic. Other antigens found in the crude cytotoxin, such as LPS, capsular polysaccharide and outer membrane

proteins, are also of potential importance as vaccine constituents and are discussed later.

The main emphasis of the work on the cytotoxin in this thesis was to produce detectable amounts of toxin from the A2 serotype and assess the potential of the product as a vaccine constituent. In the absence of purified, active toxin, detection of the cytotoxin is still based on evidence of its biological activity. Neutralisation of the toxin is also best demonstrated by inhibition of biological activity. The serotype A2 cytotoxin was standardised by the chromium-51 release assay, and rabbit antibodies raised against the crude cytotoxin were found to neutralise the toxic activity. The rabbit serum did not, however, have a high CN titre (compared to titres from "convalescent" lambs), despite the use of large amounts of crude cytotoxin (10 mg per dose) in the vaccine. This indicated either that only small amounts of the cytotoxin component were present in the crude cytotoxin or that the toxin was not particularly immunogenic. The raising of CN antibodies with the crude cytotoxin indicated, however, that this preparation may be a valuable constituent in an experimental vaccine.

Further evidence for the potential protective effects of cytotoxin neutralising antibodies was given by the demonstration that "convalescent" lamb sera and lung washings had significant CN antibody titres. In contrast, control lamb sera had negligible CN titres, although control lambs which survived to necropsy did have CN antibody titres in lung washings. These were probably due to a local response to challenge and may have been associated with the survival of these lambs. Generally, these findings indicated a correlation for CN antibodies with immunity.

Although CN antibodies were therefore considered potentially important in immunity to P. haemolytica A2, it was also considered that for complete protection vaccines would need to induce antibodies that stimulated mechanisms involved in the clearance of the live organism from the host. Complement-dependent bacterial killing of Gram-negative organisms and opsonin-mediated phagocytosis are both effector mechanisms of immunity that can be stimulated by specific antibodies in the immune host to induce clearance of organisms during infection. The susceptibility of P. haemolytica A2 to these mechanisms of immunity was therefore investigated with a view to determining their importance in protection against pneumonic pasteurellosis.

P. haemolytica A1 was reported by MacDonald *et al.* (1983) to be susceptible to antibody-mediated bactericidal activity operating via the classical pathway of complement. The role of this mechanism of immunity in protection against P. haemolytica A1 was however not established. In this thesis an *in vitro* microtitre plate assay was developed to determine whether this mechanism of immunity may be important in combating disease caused by P. haemolytica A2 infection in lambs. The assay measured the capacity of specific antibody to activate bacterial killing mechanisms. The assay was rapid, used small sample volumes and could deal with large numbers of samples in one day. As such, the assay may have application to studies on other organisms.

P. haemolytica A2 organisms were found to be susceptible to antibody-dependent bactericidal killing mechanisms and LPS was determined as a target antigen for antibodies by absorption studies. Evidence that this mechanism of immunity may be involved



in protection against pneumonic pasteurellosis caused by P. haemolytica A2 was given by the finding that sera and lung washings from "convalescent" lambs were significantly bactericidal for P. haemolytica A2 whereas similar samples from control animals were not.

Since LPS was shown to be a target antigen involved in bactericidal activity against serotype A2 organisms, this suggested that inclusion of LPS in experimental vaccines could be important in stimulating mechanisms for the clearance of biotype A organisms during infection.

Serotype T10 organisms were, however, found to be resistant to bactericidal mechanisms and this resistance appeared to be due to the presence of "smooth" type LPS, which was demonstrated by SDS-PAGE in this and other T serotypes. This was in contrast to the A serotypes which had "rough" type LPS. The resistance of the T10 serotype indicated that bactericidal mechanisms may not be able to combat the systemic type of disease caused by T serotypes and may, in fact, be a reason why the T serotypes are able to cause systemic disease in adult sheep. Septicaemic strains of other bacterial species are often found to be resistant to bactericidal activity and to possess "smooth" type LPS (Turk, 1959; Chedid et al., 1968; Rowley, 1968; Taylor, 1975; Madonna and Allen, 1981).

Another mechanism of immunity which may be important in the clearance of bacteria during infection is opsonin-mediated phagocytosis. In respiratory infections in particular, the resident BAM has been considered to be the first line of defence against infection (Reynolds and Newball, 1976). The sheep BAM may be compromised by P. haemolytica cytotoxin once infection is



established. However, in the early stages of infection, the immune animal may be able to neutralise the cytotoxin with CN antibodies and opsonic antibodies may effect rapid clearance of the organism. As such, opsonophagocytosis may be an important mechanism of immunity to stimulate by vaccination. Evidence to support this assumption was given by Donachie et al. (1986a), who found that opsonophagocytic indices in "convalescent" lambs correlated with immunity.

In this thesis, a microfiltration assay was employed for measuring the opsonic potential of sera. The filtration process was demonstrated to retain phagocytes whilst excluding extracellular bacteria. The assay was rapid, economic in reagents and capable of dealing with large numbers of samples in a day. The assay was firstly employed to characterise the role of opsonins in the phagocytosis of P. haemolytica (section 5) and later (section 8) to determine the role of opsonin-mediated phagocytosis in the protection given by an experimental vaccine against P. haemolytica A2 infection.

Both complement and antibody were found to be significantly opsonic for P. haemolytica A2. The opsonic potential of immunoglobulin was considered to be of greater importance in respect of acquired immunity, since this opsonin could potentially be stimulated by vaccination. Absorption studies indicated that LPS was not a target antigen for opsonic antibody, but antigens present in SSE preparations did absorb opsonins. Whilst anti-LPS antibodies have been implicated as opsonins for P. aeruginosa (Young, 1972), capsular antigens have been shown to be of prime importance in opsonisation of other bacteria such as S. pneumoniae

(Giebink et al., 1977) and E. coli (Van Dijk et al., 1979). Capsular antigens are a major constituent of SSE (Donachie et al., 1984b) and this may have been the antigen involved in absorption of opsonins by the SSE preparation. Outer membrane proteins are also present in SSE (fig 3.1) and whether these have a role in opsonisation is worthy of further investigation. Monoclonal antibodies against these antigens would undoubtedly help in the elucidation of their role as target antigens for opsonins. Monoclonal antibodies have been used for such purposes recently with P. haemolytica A1 (Penaredondo et al., 1988).

The phagocytic potential of PMNL was not examined because of the considerable difficulties involved in isolating sheep PMNL (Sutherland, 1985). These cells are generally considered to be capable of augmenting the phagocytic potential of resident BAM and have been implicated in the clearance of P. haemolytica A2 from infected sheep lungs (Davies and Penwarden, 1981). PMNL are usually recruited to an inflammatory site by chemotactic stimuli. The presence of P. haemolytica cytotoxin in vitro has been shown to inhibit the release of chemotaxins by bovine BAM (Markham et al., 1982) indicating another perturbing aspect of this toxin against host defence mechanisms. It is not known whether P. haemolytica antigens are chemotaxins or chemotaxinogens, but LPS, for example, has been shown to be a chemotaxinogen for other Gram-negative species (Morrison and Ulevitch, 1978).

Intracellular killing of P. haemolytica A2 by phagocytes was also not examined, but others have shown that BAM and PMNL are bactericidal for internalised P. haemolytica A1 (Maheswaran et

al., 1980; Czuprynski et al., 1987). Extracellular killing of bacteria can also be mediated by the secretion of antibacterial factors such as lysozyme (Gordon et al., 1974) and catalytic proteases and hydrogen peroxide from activated macrophages (Nathan et al., 1979).

Chemotaxis, intracellular killing and complement-dependent opsonisation are unlikely to be modifiable by vaccination but may play an important role in innate immunity against *P. haemolytica* infection.

In vitro grown *P. haemolytica* A2 organisms were found to be susceptible to antibody-mediated bacterial killing and opsonophagocytosis, and indications that these mechanisms of immunity may be important in protection were given by studies with "convalescent" lamb sera. However, bacteria are known to undergo phenotypic changes in vivo, and these changes can alter susceptibility to immune mechanisms (Penn et al., 1976). *P. haemolytica* A2 organisms were therefore grown in vivo in peritoneal chamber implants and these organisms were compared with in vitro grown organisms for their susceptibility to opsonophagocytosis and bacterial killing.

Donachie and Gilmour (1988) have recently detected changes in outer membrane protein profiles of in vivo grown organisms and in particular demonstrated the production of iron restricted proteins (IRPs) of 70 and 100 kDa. These proteins were demonstrated in organisms recovered from peritoneal chambers and this was considered as proof that these organisms expressed a different phenotype when grown in vivo.

*S. epidermidis* grown in peritoneal dialysate was also found to

express additional cell-surface proteins of 27, 39, 45, 54 and 98 kDa. The 16, 35 and 39 kDa proteins were depressed under iron-replete growth conditions in vitro, suggesting that growth of these proteins was iron-regulated (Williams et al., 1988).

In vivo grown P. haemolytica A2 were found to be as susceptible to killing by antibody and complement as were in vitro grown organisms. The LPS profile of in vivo grown organisms was similar to that found for in vitro grown organisms (section 4). This indicated that phenotypic changes had not occurred in the LPS structure in vivo, and since LPS was found to be a target antigen for such antibodies, this complemented the finding that no change in the susceptibility to antibody-mediated bacterial killing occurred. In vivo grown organisms were, however, found to be additionally susceptible to killing in the absence of exogenous antibody. The demonstration, by immunoblotting methods, that in vivo organisms had host immunoglobulin bound to them suggested an explanation for this observed additional susceptibility. The bound IgG may also have been responsible for the slightly elevated levels of phagocytosis found in the presence of the standard negative serum when compared with in vitro grown organisms under the same conditions. Bound IgG has also been detected on S. epidermidis organisms grown in peritoneal dialysate (Williams et al., 1988).

In vivo grown P. haemolytica A2 organisms were found to be susceptible to antibody-mediated phagocytosis, but to a lesser degree than in vitro grown (18 hour culture) organisms. In vivo and in vitro grown organisms were therefore compared for the amounts of capsular polysaccharide they possessed. Capsules were detected by Maneval stain but no differences were observed in the

thickness of capsule between in vivo and in vitro grown (18 and 24 hour cultured) organisms. An increase in capsule size was therefore not an explanation for the reduced susceptibility of in vivo organisms to opsonophagocytosis. Interestingly though, 6 hour in vitro grown organisms did have thicker capsules than organisms from 18 hour cultures. This is in agreement with the findings of Corstvet et al. (1982) who had similar findings with P. haemolytica A1 organisms from 6 and 18 hour cultures. The thicker capsule found on organisms from earlier cultures of P. haemolytica A1 may explain why these organisms were found to be less susceptible to phagocytosis (Berrgren et al., 1981).

This thesis has concentrated on examining humoral mechanisms of immunity to P. haemolytica A2 despite the suggestion by Wells et al. (1979) that humoral immunity was unimportant in protection against ovine pasteurellosis, and their suggestion as a result that CMI may be the most important mechanism of defence in this disease. More recently, and in contrast, Donachie et al. (1986a) found humoral immunity to be important in the immunity of "convalescent" lambs, whereas no CMI response was detectable, at least by the lymphocyte transformation test.

To determine further whether humoral mechanisms were of prime importance in immunity to P. haemolytica A2 infection, passive protection experiments were carried out with immune sera. These sera were shown to be highly protective against experimental infection and variously stimulated the effector mechanisms of immunity studied in this thesis. This demonstrated that humoral immunity was of prime importance in immunity. These studies also indicated that parenteral humoral immunity could be transudated



into the lung and protect against respiratory infection, suggesting that serum antibody levels should correlate with immunity, and that the ability to monitor these antibody levels with in vitro assays may be an important means by which the efficacy of vaccines can be assessed.

Since the crude cytotoxin preparation from P. haemolytica A2 was found to stimulate CN antibodies, and these antibodies had been correlated with protection in "convalescent" lambs, it was decided to test the protective capacity of this preparation in the form of a novel vaccine against experimental P. haemolytica A2 infection in lambs. In designing a vaccine based on the cytotoxin it was however decided to take into account the findings of others who had developed toxin based vaccines. Protective vaccines against cholera were shown to be most efficacious when both cholera toxoid and the main protective somatic antigens of V. cholerae were included in the vaccine. Svennerholm and Holmgren (1976) found that antitoxin and anti-LPS antibodies combined synergistically to give optimal protection in a rabbit infection model. This strategy of combining toxoids with somatic antigens has apparently also been adopted in the past for vaccines against clostridial diseases of sheep (Outteridge, 1985). It was therefore considered that the combination of P. haemolytica cell-surface antigens, in the form of SSE, with the crude cytotoxin preparation might constitute the most efficacious vaccine against P. haemolytica A2. By vaccination with this formulation, the CN antibodies induced would conceivably combat the leucocytic effects of the cytotoxin, whilst antibodies produced against cell-surface antigens may promote antibody-mediated complement-dependent bactericidal activity and/or opsonise bacteria to enhance phagocytosis.



As the results indicated however, the crude cytotoxin was highly protective even without the addition of SSE. The SSE alone was poorly protective, confirming the previous findings of Gilmour et al. (1983). The crude cytotoxin preparation probably contained sufficient cell-surface antigens for it not to require the addition of SSE. The presence of cell-surface antigens in the crude cytotoxin was detected in section 3 of this thesis. The presence of these antigens probably explains why the crude cytotoxin promoted both cytotoxin neutralisation and bactericidal activity, both of which correlated with immunity. It was particularly interesting that the crude cytotoxin preparation stimulated higher levels of bactericidal activity than the SSE vaccine. Both of these preparations contained LPS, but perhaps these results indicate that the LPS is presented in a more immunogenic form in the crude cytotoxin. The crude cytotoxin possibly presented a more natural form of LPS since, unlike the SSE preparation it is not treated by chemicals. The poor immunogenicity of Westphal-purified LPS for mice and rabbits has made it of questionable value as a vaccine antigen against organisms such as P. multocida (Rebers et al., 1980). LPS from P. multocida was found to be most immunogenic when presented in a more native form: as a complex with proteins (Ganfield et al., 1976), or when complexed with ribosomes (Phillips and Rimler, 1984).

The finding that the crude cytotoxin was highly protective without the addition of cell-surface antigen extracts is perhaps of particular importance in the manufacture of vaccines, in that production is much simplified.

Vaccines comprising crude cytotoxin, crude cytotoxin plus SSE

or SSE alone failed to stimulate opsonic antibodies or IHA titres. This is in contrast to the response of immune, "convalescent" lambs which did produce opsonic antibodies (Donachie *et al.*, 1986a; section 5 this thesis) and IHA titres (Donachie, personal communication). An explanation for the inability of sub-particulate vaccines to induce opsonic antibodies may lie in the findings of Adlam *et al.* (1987). These authors have characterised the chemical composition of the *P. haemolytica* A2 capsular polysaccharide and found it to be an  $\alpha$ -2-8-linked polymer of N-acetylneuraminic acid. Traces of  $\alpha$ -1-4-linked dextran were also found. Both of these polymers are poorly immunogenic and if the capsule of *P. haemolytica* is an important target for opsonic antibodies (discussed in section 5), this may explain the inability of sub-particulate vaccines to induce opsonophagocytosis of *P. haemolytica* A2. The N-acetylneuraminic acid polymer of *P. haemolytica* A2 is identical to the capsular polymers of *N. meningitidis* group B (Bhattacharjee *et al.*, 1975) and *E. coli* K1 (McGuire and Binkley, 1964), and it has been noted that the *N. meningitidis* group B capsule is poorly immunogenic when included in sub-particulate vaccines used against meningitis in children (Wyle *et al.*, 1972). A possible explanation for this poor immunogenicity came from Finne (1982) and Hoffman *et al.* (1982) who found a glycoprotein in neuronal cell membranes with a sialic acid polymer which was similar to the *E. coli* K1 capsular polysaccharide. This suggests a mechanism of immune avoidance by host mimicry (Jann and Jann, 1985).

Some similarities in the protective response following vaccination to *N. meningitidis* group B in children and *P.*

haemolytica in lambs can also be seen. Capsular extracts were found to be highly protective against meningitis caused by N. meningitidis groups other than group B. In P. haemolytica infections of lambs, SSE vaccines, which include capsular antigens, were protective against serotypes A1, A6 and A9 and raised IHA antibody titres, which are a measure of the immune response to capsule (Gilmour et al., 1983). In contrast, SSE vaccines did not protect well against serotype A2 infection and IHA titres were not induced.

Protection against N. meningitidis group B now seems likely to depend upon the use of protective cell-surface proteins (Frasch, 1983). In this thesis protective antigens which were an alternative to the capsule of P. haemolytica A2 were found to be in a crude cytotoxin preparation. This preparation induced CN antibodies which an SSE preparation did not, and further, stimulated significantly higher bactericidal activity than did SSE antigens. These immune mechanisms appear therefore to represent alternative means of protection against the A2 serotype, and they compare in terms of efficacy with the capsular-based immunity which is satisfactory for other serotypes of P. haemolytica. A cytotoxin-based vaccine may, however, protect against other serotypes equally well, and since the cytotoxin is cross-reactive amongst P. haemolytica serotypes (Shewen and Wilkie, 1983b), cross-protection may be available from a single-serotype preparation.

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Publications Arising From This Thesis

## A Rapid Micro-Method for the Study of Antibody-Mediated Killing of Bacteria, with Specific Application to Infection of Sheep with *Pasteurella haemolytica*

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### ABSTRACT

Sutherland, A.D., 1988. A rapid micro-method for the study of antibody-mediated killing of bacteria, with specific application to infection of sheep with *Pasteurella haemolytica*. *Vet. Microbiol.*, 16: 263-271.

A micro-titration plate bactericidal assay was developed to measure complement-dependent antibody-mediated killing of *Pasteurella haemolytica*. Sera and lung washings from specific pathogen-free (SPF) lambs convalescent from a challenge with live, virulent *P. haemolytica* were bactericidal in the presence of complement. Similar samples from naive SPF lambs had no such activity. Purified IgG derived from a convalescent lamb serum was as bactericidal as the whole serum. Absorption of convalescent serum with lipopolysaccharide from *P. haemolytica* abolished bactericidal activity, suggesting that this antigen may be a target for antibody in the bactericidal complex.

### INTRODUCTION

In the U.K., *Pasteurella haemolytica* is the organism most commonly associated with acute pneumonia in sheep (Gilmour, 1980). *P. haemolytica* can be divided into two biotypes, A and T (Smith, 1961), and within these biotypes 15 serotypes have been identified (Fraser et al., 1982b). Of 1198 isolates from cases of ovine pasteurellosis (Fraser et al., 1982a) 38% were serotyped as Biotype A Serotype 2 (A2), which therefore constitutes the predominant serotype implicated in ovine pasteurellosis in Britain.

Experimental vaccines based on sodium salicylate extracts (SSE) of the *P. haemolytica* cell wall have been developed (Donachie et al., 1984). These were generally of good efficacy, but those prepared from Serotype A2 were less protective against homologous experimental challenge than those from other serotypes, and offered no protection against heterologous challenge (Gilmour et

al., 1983). However, strong immunity to the A2 serotype is possible, as was demonstrated recently when specific pathogen-free (SPF) lambs, convalescent from a virulent A2 serotype challenge, were shown to be completely refractory to a subsequent homologous challenge (Donachie et al., 1986).

In studies relating to protection against pneumonic disease, it is important to define the functional mechanisms of immunity which operate both systematically and in the lung. It is also desirable to determine which antigen(s) stimulate those mechanisms of immunity after vaccination. *P. haemolytica* A1 was shown to be susceptible to the bactericidal activity of antibodies in adult cattle sera when these antibodies were combined with complement (Macdonald et al., 1983). Antibody- and complement-mediated bacterial killing may, therefore, be a mechanism contributing to the prevention or suppression of *P. haemolytica* infection in the sheep.

This paper describes an improved, rapid micro-method which enables large numbers of samples to be screened for antibody- and complement-mediated bactericidal activity. The method was used to assay bactericidal antibodies in sera and lung washings of lambs which were either resistant or susceptible to experimental challenge with *P. haemolytica*. Finally, a standard convalescent serum was absorbed with selected antigen preparations in an attempt to identify target antigen(s) involved in the bactericidal complex.

#### MATERIALS AND METHODS

##### *Bacterial suspension*

A few separate colonies from a cloned isolate (T884) of *P. haemolytica* A2 were removed from a 7% sheep blood agar (SBA) plate culture and inoculated into 10 ml of nutrient broth (NB). After incubation at 37°C overnight, 200 µl of culture were transferred to 50 ml of NB and grown at 37°C for 3–4 h to obtain a log-phase culture, which was then centrifuged at 11 000 × *g* for 5 min at room temperature (Taylor, 1983). The resultant bacterial pellet was washed once in modified barbitol buffer (MBB) (Oxoid, U.K.) to remove residual NB, which has been shown to be anti-complementary (Muschel and Treffers, 1956). The bacterial inoculum was finally adjusted with MBB to the required number of colony-forming units per millilitre (cfu ml<sup>-1</sup>) by comparing its optical density at 420-nm wavelength with a standard curve of cfu ml<sup>-1</sup> against optical density. The actual bacterial number was confirmed by retrospective plate counting on SBA.

##### *Sera and lung washings*

These samples were collected as previously described (Donachie et al., 1986) from two groups of animals which, in brief, were treated as follows. Seven SPF

lambs were immunised by challenge (Day 0) with a virulent strain (T884) of *P. haemolytica* A2, followed by antibiotic treatment to prevent death. These animals ("convalescent" group) were infected with parainfluenza type 3 virus (P13 virus) at Day 27 and re-challenged with the homologous strain of *P. haemolytica* A2 at Day 34. Seven naive lambs (control group) were challenged on the second occasion only with P13 virus and *P. haemolytica* A2 (Days 27 and 34, respectively). Only 3 animals survived until necropsy and these provided control samples for the assay. All animals were necropsied on Day 41.

Serum from a convalescent lamb was included in each assay as a standard positive control. The negative control was fetal bovine serum (FBS). Standards and test samples were heat inactivated at 56°C for 30 min before assay to destroy endogenous complement.

Purified Immunoglobulin G (IgG) was prepared from aliquots of the standard positive serum by immunoaffinity chromatography. A column consisting of a 9-ml bed-volume of cyanogen bromide-activated sepharose 4B (Pharmacia Ltd., Uppsala, Sweden) ligated with 50 mg of pig IgG anti-sheep IgG (Fc fraction) was used for purification. The purity of the recovered IgG was confirmed by immunoelectrophoresis against rabbit anti-whole-sheep serum. The IgG concentration was determined by centrifugal analyser (Allied Instrumentation Laboratories, Warrington, U.K.) and adjusted to correspond to the original serum IgG concentration.

Other 1-ml aliquots of the standard positive serum were absorbed with 1 mg of either SSE or lipopolysaccharide (LPS) of *P. haemolytica* A2 cell wall. The LPS was purified by the method of Westphal et al. (1952) from cells grown in nutrient broth at 37°C for 18 h.

#### *Sources of standard complement*

Sera from naive SPF lambs or fetal calves were collected and stored in aliquots at -70°C until used. Each batch of serum was tested before use for the absence of bactericidal antibodies and the presence of sufficient complement to cause 100% bactericidal activity when used in conjunction with the standard positive control serum.

#### *Assay procedure*

Initially, optimal conditions for the demonstration of antibody and complement activity were determined with respect to bacterial concentration and incubation period.

The assay was performed in 96-well, tissue culture-grade, flat-bottomed micro-titre plates (NUNC, Denmark) into which reactants were simultaneously delivered to 12 wells with a multi-channel pipette. Triplicate assay wells received 20 µl of each standard serum or test sample followed by 100 µl of bac-

terial inoculum. Assay suspensions were incubated for 15 min at room temperature to allow antibody-bacterium interaction to occur. Standardised serum complement was then added in 80- $\mu$ l volumes to each assay well, except when evaluating the effect of antibody on *P. haemolytica* in the absence of complement, when 80  $\mu$ l of heat-inactivated FBS was added.

Assay plates were covered with a sterile adhesive plastic film (Flow Laboratories, Irvine, U.K.) and incubated at 37°C for the required period. Generally, after incubation, 10- $\mu$ l samples of each triplicate suspension were removed with a multi-channel pipette and inoculated onto a 12  $\times$  12-cm SBA plate (Sterilin Ltd., Feltham, U.K.). All 96 wells could be inoculated onto one SBA plate. When bacterial counts were too high for direct plate counting, as when the assay was being evaluated, sequential logarithmic dilutions of each suspension were made in MBB diluent in microtitre plates before counting. After incubation of SBA plates at 37°C overnight, the mean number of cfu ml<sup>-1</sup> was calculated from each triplicate.

The mean percent of bacterial inoculum which was killed (%K) in each suspension was calculated from the formula  $\%K = 100 \times 1 - (\text{the mean number of cfu ml}^{-1} \text{ after incubation} / \text{the mean number of cfu ml}^{-1} \text{ before incubation})$ .

The %K for each lung washing was adjusted by a correction factor (Donachie et al., 1986) to take account of the extent to which each sample had been concentrated before assay.

## RESULTS

### *Optimisation of the bactericidal assay*

The micro-titre plate assay could be performed rapidly with little variation in counts between sample triplicates. The standard positive and negative sera gave repeatable results (100 and 0%K, respectively) from day to day.

The standard positive serum killed 100% of a bacterial suspension containing  $3.4 \times 10^3$  cfu ml<sup>-1</sup>, but only in the presence of complement. No killing occurred when complement was replaced with heat-inactivated FBS. This bactericidal capacity rapidly diminished with dilution of the standard positive serum, and was abolished at 1/32 (Fig. 1). The standard negative serum was not bactericidal at any dilution (Fig. 1).

The standard positive serum was tested with various concentrations of *P. haemolytica* (Fig. 2). The activity of the serum remained >85% for *P. haemolytica* concentrations between  $3.4 \times 10^3$  and  $3.4 \times 10^5$  cfu ml<sup>-1</sup>, but thereafter decreased rapidly with increasing bacterial concentration. A bacterial concentration of  $3 \times 10^3$  cfu ml<sup>-1</sup> (equivalent to an inoculum of  $6.0 \times 10^3$  cfu ml<sup>-1</sup>) was therefore selected.

Bacterial killing by the standard positive serum was apparent after 2 min of



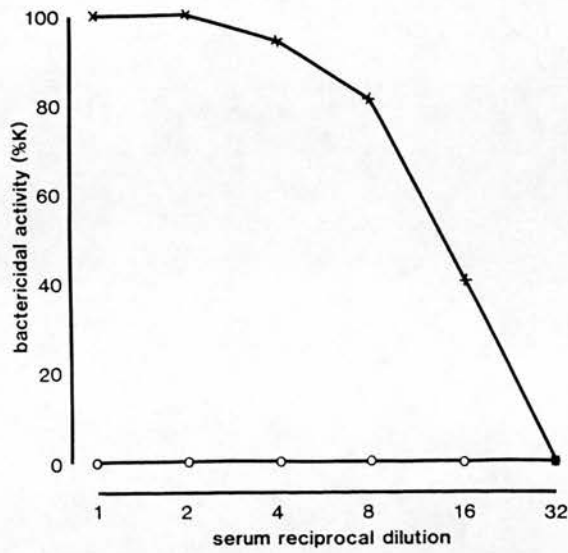


Fig. 1. Titration of the bactericidal activity of a convalescent (x—x) and a naive (o—o) specific pathogen-free lamb serum.

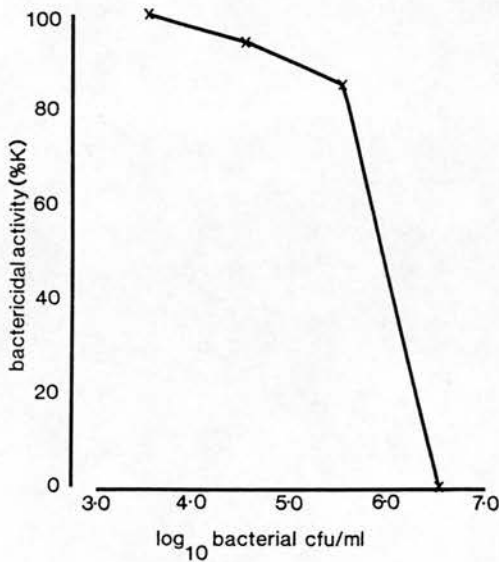


Fig. 2. The bactericidal activity of a convalescent lamb serum for varying titres of *P. haemolytica*.

incubation (97%K) and had increased to 100%K by 15 min. An incubation period of 30 min was therefore selected.

Fresh FBS was as effective a source of complement as naive SPF lamb serum, but less convenient to obtain. Complement source sera were used undiluted

since the bactericidal capacity of sera decreased rapidly in the presence of a diluted complement source, e.g., 100% killing by one serum in the presence of a neat concentration of the complement source was reduced to 86% when the complement source was diluted 1 in 2. Heat inactivation of the complement source completely abolished bactericidal activity, demonstrating that complement was essential. The complement source was not bactericidal in the presence of the standard negative serum, indicating that specific antibody was also essential for bacterial killing. That antibody was the essential component in the standard positive serum was confirmed by the use of immunoaffinity-purified IgG isolated from this serum. This proved to be as bactericidal (100%K) as the complete serum when used at an equivalent concentration ( $2.27 \text{ g l}^{-1}$ ).

*The bactericidal capacity of convalescent and naive SPF lamb sera and lung washings*

Sera taken from the "convalescent" group lambs on Day 0 (pre-immunising challenge) had no bactericidal activity, but when collected on Days 34 and 41 had group mean activities of 92.1 and 89.8%K, respectively (Table I). Further, all but one lung washing obtained at necropsy (Day 41) from these "convales-

TABLE I

The bactericidal activity (%K) of sera and lung washings from "convalescent" lambs<sup>a</sup>

Animal	%K of serum			%K of lung washings <sup>b</sup> (at necropsy)
	Pre-first <sup>c</sup> immunising challenge (Day 0)	Post-first challenge (Day 34)	Post-second challenge (necropsy, Day 41)	
1	0	97.6	95.5	13.2
2	0	NA <sup>d</sup>	98.5	18.0
3	0	93.2	94.5	0
4	0	99.5	99.2	27.0
5	0	99.0	99.6	9.8
6	0	98.0	98.4	11.6
7	0	65.5	42.7	11.3
Group mean ( $\pm$ S.E.)	0	92.1 ( $\pm$ 5.4)	89.8 ( $\pm$ 7.9)	12.99 ( $\pm$ 3.1)

<sup>a</sup>Bactericidal activity was not detectable in sera or lung washings of control lambs.

<sup>b</sup>The bactericidal activity of lung washings taken at necropsy was adjusted by a correction factor to return all samples to a unit volume of the original harvest.

<sup>c</sup>By challenge with live organisms, followed by prevention of fulminating disease by antibiotic therapy.

<sup>d</sup>NA = not available.

cent" group lambs also contained bactericidal activity, although this was less than in their sera (Table I). Conversely, no activity was detected in sera or lung washes of 3 control group lambs nor were these lambs protected against *P. haemolytica* A2.

#### *Target antigens implicated in the bactericidal assay*

Aliquots of the standard positive serum were absorbed with either SSE of *P. haemolytica* A2 cell wall or LPS. Absorption with LPS completely abolished bactericidal activity. The SSE reduced activity to 21%K.

#### DISCUSSION

The bactericidal assay described in this paper was rapid, gave little variation amongst replicates and was comparable on a day-to-day basis. Titration of the immune serum and bacterial inoculum indicated that assay results were dependent upon both antigen and antibody titre. The most suitable conditions for assay of SPF lamb sera or lung washings were 20  $\mu$ l of neat serum and  $3 \times 10^3$  cfu ml<sup>-1</sup> of *P. haemolytica*.

Serum complement was not bactericidal for *P. haemolytica* in the absence of antibody, indicating that *P. haemolytica* could not activate the alternative complement pathway. This result is in agreement with the findings of Macdonald et al. (1983) who showed that *P. haemolytica* was not killed in the absence of a functional classical complement pathway. Also, the standard positive serum was not bactericidal unless in combination with complement, indicating that bactericidal activity was due to an antibody-complement complex.

Purification proved that IgG was the bactericidal component in the standard positive serum. The bactericidal activities of IgA and IgM against *P. haemolytica* remain to be investigated in this system. Previous studies have shown that IgM is more active than IgG in bactericidal assays (Robbins et al., 1965; Bjornson and Michael 1970; Schulkind et al., 1972), although the activity of IgG has been found in rabbit serum to increase during sequential immunisation (Pike and Chandler, 1971). Also, IgG is predominantly produced in an anamnestic response and is the major antibody class found in the lower respiratory tract of sheep (Gorin et al., 1979). It is doubtful whether IgA can activate bactericidal activity via the classical pathway (Sirotak et al., 1976) and it has even been suggested that IgA may block bactericidal activity against *P. haemolytica* (Macdonald et al., 1983). The immunoglobulin class meriting most attention following vaccination or development of pneumonia would, therefore, seem to be IgG.

SPF lambs convalescent from *P. haemolytica* A2 infection were recently shown to be immune to subsequent challenge with the homologous serotype

(Donachie et al., 1986). In this report, the sera and all but one of the lung washings of those convalescent animals were found to exhibit bactericidal activity against *P. haemolytica* A2. In contrast, samples from untreated control animals which were susceptible to the *P. haemolytica* challenge had no bactericidal activity. The presence of bactericidal antibodies in the sera and lung washings of the convalescent lambs, therefore, correlated with resistance to *P. haemolytica*. Whether sufficient complement is available in the lung for bactericidal activity to occur is uncertain, but Burrells (1986) has shown that the C3 component of complement increases in the sheep lung during PI3 virus/*P. haemolytica* infection. The possibility that complement behaves as an acute-phase reactant in disease has been suggested elsewhere (Atkinson and Frank, 1980). Because the recovery of lung washings involved dilution of the lung fluids, bactericidal activities of sera and lung washings were not comparable, therefore, although the bactericidal capacity of the lung washings was lower than their respective sera, this may not necessarily reflect the situation in vivo. The lungs of these convalescent lambs may have constituted as bactericidal an environment for *P. haemolytica* as their blood. It is perhaps more relevant to note that control lambs had no detectable bactericidal antibody in their lung washings at necropsy.

In attempts to determine which antigen was the target for antibodies involved in the bactericidal assay, absorption with LPS was found to abolish the bactericidal activity of the standard positive serum. Previous authors have described LPS as being the antigen receptor for the bactericidal antibodies directed against other gram-negative bacteria (Glynn and Ward, 1970; Tramont et al., 1974). An SSE preparation of *P. haemolytica* A2 also reduced the activity of bactericidal antibodies. This crude extract contained small amounts of LPS and this was probably responsible for the reduction in bactericidal activity, although other cell-surface antigens such as outer membrane proteins (Tramont et al., 1974) and phage receptors (Gabay, 1977), have been implicated as targets for bactericidal antibodies. Separation of SSE into its components may, therefore, reveal additional target antigens. The bactericidal assay described here will be useful in the understanding of the mechanisms of immunity against *P. haemolytica* and in monitoring the ability of vaccines to produce bactericidal activity both systematically and in the lung. This assay may have similar applications for the study of bactericidal activity against other gram-negative bacteria.

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## A Crude Cytotoxin Vaccine Protects Sheep Against Experimental *Pasteurella haemolytica* Serotype A2 Infection

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### ABSTRACT

Sutherland, A.D., Donachie, W., Jones, G.E. and Quirie, M., 1989. A crude cytotoxin vaccine protects sheep against experimental *Pasteurella haemolytica* Serotype A2 infection. *Vet. Microbiol.*, 19: 175-181.

Three vaccines containing *Pasteurella haemolytica* serotype A2 antigens were tested for their ability to protect sheep against a homologous challenge. A crude cytotoxin preparation in combination with a sodium salicylate extract (SSE) or crude cytotoxin alone were highly protective (98 and 86%, respectively), whereas SSE alone was poorly (47%) protective. These findings indicated that the crude cytotoxin was an essential component of a protective vaccine. Protection correlated with serum cytotoxin-neutralising (CN) titres and bactericidal activity, which were stimulated by antigens in the crude cytotoxin preparation.

### INTRODUCTION

*Pasteurella haemolytica* is the causative organism of ovine pneumonic pasteurellosis in Britain (Gilmour, 1980) and biotype A serotype 2 (A2) is the most frequently isolated serotype (Fraser et al., 1982). However, A2 was the least immunogenic serotype of *P. haemolytica* when sodium salicylate extract (SSE) vaccines were evaluated against homologous challenge in lambs and significant protection was obtained only when heat-killed organisms were included (Gilmour et al., 1983). Subsequently, Donachie et al. (1986) showed that convalescent lambs were completely protected against a further homologous A2 challenge, which suggested that effective immunisation against the A2 serotype was possible and that the protective antigens were either absent from or present in insufficient amounts in the SSE vaccines tested.

Ovine isolates of *P. haemolytica* produce an extracellular cytotoxin which is lethal for ovine leucocytes (Sutherland et al., 1983; Sutherland, 1985) and which may be an important pathogenic determinant in ovine pneumonic pasteurellosis. It was considered, therefore, that a toxoid might provide protection against *P. haemolytica* A2. Toxin-neutralising activity in itself may not, how-



ever, be sufficient. Clostridial toxoid vaccines were poorly protective in sheep unless they additionally included somatic antigens which stimulated the clearance of organisms (Outteridge, 1985); therefore two vaccines were prepared, one of which contained a crude form of cytotoxin, the other the crude cytotoxin plus SSE. These toxin-based vaccines were compared with an SSE vaccine similar to that described previously (Gilmour et al., 1983). Serum antibody responses were measured by an enzyme-linked immunosorbent assay (ELISA) for antibodies to SSE cell surface antigens (Donachie et al., 1986), a cytotoxin-neutralisation (CN) test and a bactericidal test (Sutherland, 1988), and results from these tests were compared for correlation with protection against *P. haemolytica* A2 infection.

#### MATERIALS AND METHODS

##### *Production of a cytotoxin preparation*

Crude cytotoxin was prepared as described previously (Sutherland and Redmond, 1986). *P. haemolytica* A2 cultures in dialysis sacs submerged in 500-ml volumes of nutrient broth were incubated for 24 h at 37°C. Sac contents were centrifuged at 10 000×g for 20 min to pellet bacteria and the culture supernatant fluid, containing cytotoxin, was filter sterilised and lyophilised after exhaustive dialysis against distilled water.

##### *Production of SSE*

The SSE was prepared by the method of Gilmour et al. (1983) with the additional step that bacterial cell pellets were washed once in phosphate-buffered saline (pH 7.2) to minimise contamination of the extracts with extracellular cytotoxin.

##### *Formulation of vaccines*

Vaccines consisted of 9% alhydrogel adjuvant (Miles Laboratories) containing lyophilised antigen resuspended in distilled water and were formulated as follows: (i) 10 mg ml<sup>-1</sup> of crude cytotoxin; (ii) 2.5 mg ml<sup>-1</sup> of SSE; (iii) 10 mg ml<sup>-1</sup> of crude cytotoxin plus 2.5 mg ml<sup>-1</sup> of SSE.

##### *Experimental animals*

Lambs were caesarian derived, colostrum deprived and maintained in isolation. They were fed sterilised cows' milk, hay and concentrates, and designated specific pathogen-free (SPF) lambs.

### *Experimental infections*

The experimental infection of lambs with *P. haemolytica* A2 was preceded by infection with parainfluenza virus Type 3, a method which consistently induces severe pneumonia in SPF lambs (Sharp et al., 1978). PI3 virus was administered intratracheally (8 ml) and intranasally (2 ml). The titre of the virus was  $1 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>. Infection with *P. haemolytica* A2 was achieved by exposure for 15 min to an aerosol of strain X205A at  $\sim 4 \times 10^7$  colony-forming units l<sup>-1</sup> and was administered 7 days after infection with PI3 virus.

### *Experimental design*

Three-week-old SPF lambs were allocated randomly into four groups. The SSE vaccine which had been previously tested for efficacy (Gilmour et al., 1983) and the control group were allotted eight animals. The untested cytotoxin and cytotoxin plus SSE vaccines were allotted 14 animals to ensure that sufficient animals were available for statistical comparison between the vaccine groups. One animal in each of the cytotoxin, SSE and control groups died prior to experimental infection with PI3 virus. Three groups were vaccinated on Day 0 and Day 28 with 1-ml doses of each vaccine, while one group remained unvaccinated. All animals were given PI3 virus on Day 35 and an aerosol of *P. haemolytica* A2 on Day 42. Lambs were bled for serum on Day 42. Clinical examinations were carried out for 6 days after *P. haemolytica* infection and surviving animals were necropsied on Day 49.

### *Clinical and pathological examinations*

A clinical, microbiological and pathological assessment of the response of each lamb was made according to the method of Jones et al. (1989).

### *Measurement of cytotoxin potency and serum CN antibody titres*

The <sup>51</sup>Cr-release cytotoxicity assay was a modification of that described by Shewen and Wilkie (1982) and results were calculated by a modification of the method of Gentry et al. (1985). Briefly, sheep bronchoalveolar macrophages (BAM) were isolated, as described previously by Burrells (1985), and resuspended at  $4 \times 10^7$  viable BAM ml<sup>-1</sup> in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine, 3 mM Hepes buffer, 2% sodium bicarbonate, adjusted to pH 7.4 with 1 M NaOH (complete RPMI medium). BAM counts and viability were assessed by trypan blue exclusion in an improved Neubauer haemocytometer. Sodium chromate <sup>51</sup>Cr isotope was added to the BAM suspension at 50 µCi ml<sup>-1</sup> and incubated at 37°C for 1 h, after which cells were sedimented and washed three times in

Hanks basic salt solution by centrifugation at  $100\times g$  for 10 min at room temperature. The BAM suspension was finally resuspended in complete RPMI medium at  $2\times 10^6$  viable BAM  $\text{ml}^{-1}$ .

The potency of the crude cytotoxin preparation used for vaccination and serology was assessed by titration. Lyophilised cytotoxin was resuspended at  $20\text{ mg ml}^{-1}$  in complete RPMI medium and duplicate  $100\text{-}\mu\text{l}$  volumes were titrated by doubling dilution in sterile, tissue culture grade flat-bottomed microtitre plates (Nunc, Denmark). Complete RPMI medium ( $100\text{ }\mu\text{l}$ ) was added to the titrated cytotoxin to replace the volume of test serum added when determining CN titres. Finally, BAM ( $100\text{ }\mu\text{l}$ ) were added to each well, including spontaneous release control wells containing  $200\text{ }\mu\text{l}$  of complete RPMI medium and total release control wells containing  $200\text{ }\mu\text{l}$  of 0.1% Triton X-100 (Sigma). Plates were sealed with sterile plastic film (Flow Laboratories, Irvine, Scotland) and incubated at  $37^\circ\text{C}$  for 3 h. BAM were then sedimented by centrifugation of the plates at  $100\times g$  for 10 min, after which  $100\text{ }\mu\text{l}$  of supernatant fluid was removed from each well for counting in a gamma counter. The percent specific cytotoxicity for each supernatant was calculated from the formula:

Percent specific cytotoxicity = 100

$$\times \frac{\text{counts in sample} - \text{counts in spontaneous release control}}{\text{counts in total release control} - \text{counts in spontaneous release control}}$$

The end-point titre of the cytotoxin sample was taken as the final concentration of toxin which gave a percent specific cytotoxicity  $> 10\%$ .

The CN titre was taken as the highest serum dilution neutralising cytotoxic activity by  $> 80\%$  and was calculated by the formula:

$$100 \times \frac{\text{the percent specific toxicity of the cytotoxin} - \text{the percent specific toxicity of the cytotoxin in the presence of test serum}}{\text{the percent mean toxicity of the cytotoxin in the presence of a negative control serum}}$$

#### *Bactericidal assay and ELISA*

These assays were performed as previously described [Sutherland (1988) and Donachie et al. (1986), respectively]. The bactericidal capacity of serum samples was calculated as the mean percentage of bacterial inoculum killed (%K) in triplicate assay suspensions.

#### *Statistical analysis*

The Mann-Whitney ranking test was used to determine the significance of differences between group data. Correlation tests were performed by the Spearman ranking test for non-parametric data.

## RESULTS

The end-point titre of the crude cytotoxin used for vaccination was at a concentration of  $2.5 \text{ mg ml}^{-1}$ .

A high degree of protection was afforded to animals vaccinated with crude cytotoxin both with and without the addition of SSE, while SSE-vaccinated animals were protected significantly less ( $P < 0.01$ ) than the cytotoxin plus SSE group (Table 1).

All vaccinated animals had serum antibodies to *P. haemolytica* when measured by SSE-ELISA, while unvaccinated animals had none (Table 2). The mean CN titres and bactericidal capacities of sera from the cytotoxin and cytotoxin/SSE vaccine groups were significantly ( $P < 0.001$ ) greater than those of the SSE vaccine and unvaccinated control groups. Only two animals of the SSE vaccine group had serum CN activity, both of low titre, indicating that the SSE antigen contained negligible amounts of cytotoxin.

Serum CN titres and bactericidal capacities of experimental lambs corre-

TABLE 1

Group means of the experimental disease indices and percentage protection of different *P. haemolytica* vaccines

Vaccine group (n)	Deaths	Disease index <sup>1</sup>	Percent protection <sup>2</sup>
Cytotoxin (7)	1	7	86
SSE (13)	6	26	47
Cytotoxin/SSE (14)	0	1	98
Unvaccinated (7)	7	50	0

<sup>1</sup>Disease index = the sum of the clinical, consolidated lesion, *P. haemolytica* isolation index and pleurisy scores (Jones et al., 1989).

<sup>2</sup>Percent protection =  $[1 - \text{disease index of treated group} / \text{disease index of unvaccinated group}] \times 100$ .

TABLE 2

Group means of the reciprocal antibody titres in post-vaccinal sera measured by SSE-ELISA, CN and bactericidal assay

Vaccine group	SSE-ELISA	CN assay	Bactericidal assay (%K)
Cytotoxin	5360 ( $\pm 3452$ )	15.1 ( $\pm 4.3$ )	100 ( $\pm 0.3$ )
SSE	7171 ( $\pm 4179$ )	0.1 ( $\pm 0.1$ )	77 ( $\pm 4.0$ )
Cytotoxin/SSE	3819 ( $\pm 6624$ )	38.7 ( $\pm 10.6$ )	96 ( $\pm 2.5$ )
Unvaccinated	0	0.0	38 ( $\pm 5.8$ )

Figures in parentheses are the standard error of the mean.

lated inversely ( $P < 0.001$ ) with the severity of disease measured by an index taking account of clinical, pathological and microbiological parameters.

#### DISCUSSION

In this study, a vaccine containing crude cytotoxin plus SSE was 98% protective against experimental infection of lambs with *P. haemolytica* A2 and the protection afforded by the cytotoxin alone (86%) was only slightly less. However, the protection (47%) given by the SSE vaccine was significantly ( $P < 0.01$ ) poorer and similar to values found previously (Gilmour et al., 1983). Serological analysis revealed no significant difference in the mean %K of groups given crude cytotoxin or crude cytotoxin plus SSE vaccines, whereas the SSE vaccine group had a significantly lower mean %K. The antigens in the crude cytotoxin thus stimulated bactericidal activity more effectively than SSE. Similar ELISA titres in all groups showed there was no significant difference in levels of antibodies to SSE cell surface antigens. Therefore, it was concluded that SSE was not an additional requirement for protective cytotoxin-based vaccines. This is in contrast to clostridial toxoid vaccines (Outteridge, 1985) which require the addition of somatic antigens, usually in the form of bacterins. With crude cytotoxin vaccines, bactericidal activity is probably induced by contaminant lipopolysaccharide (LPS) since antibodies involved in the bactericidal activity of immune serum can be absorbed by the addition of LPS (Sutherland, 1988). The presence of LPS in crude cytotoxin has been detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Sutherland, 1986) and the limulus amoebocyte lysate assay (A.D. Sutherland, unpublished results, 1987).

Since the partially protective SSE vaccine produced negligible CN titres and low bactericidal activity, either CN antibodies or high bactericidal capacity, or both, are probably required for significant protection. Both these variables were significant correlates of immunity ( $P < 0.001$ ) and were stimulated by the crude cytotoxin vaccine.

Whether protection can be ascribed to either CN or bactericidal antibodies alone can only be determined if different antigens are responsible for inducing these antibodies, and separation and identification of these can be achieved. However, the most efficient vaccine formulation for *P. haemolytica* may include those antigens that induce both CN and bactericidal antibodies as found in the crude cytotoxin preparation. In addition, inclusion of antigens that stimulate opsonising antibodies for phagocytosis may be beneficial for protection since these correlate with immunity in animals convalescent to *P. haemolytica* A2 (Donachie et al., 1986).



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## Protection of Lambs Against Experimental Pneumonic Pasteurellosis by Transfer of Immune Serum

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### ABSTRACT

Jones, G.E., Donachie, W., Sutherland, A.D., Knox, D.P. and Gilmour, J.S., 1989. Protection of lambs against experimental pneumonic pasteurellosis by transfer of immune serum. *Vet. Microbiol.*, 20: 59-71.

Passive protection of specific pathogen-free lambs against experimental pasteurellosis was achieved using antisera from conventionally reared sheep which were either convalescent from experimental pneumonia or inoculated with *Pasteurella haemolytica* A2 vaccines. The complete immune sera, or immunoglobulin-rich fractions prepared from them, when administered separately or together provided 94-100% protection of recipients compared to control lambs.

Antibodies to *P. haemolytica* in donor sera were quantified by anti-sodium salicylate extract (SSE) and anti-lipopolysaccharide (LPS) ELISA, bactericidal assay, cytotoxin neutralization and indirect haemagglutination. The anti-SSE ELISA titres correlated best with protective efficacy and could be used to measure antibody in recipient lambs immediately before challenge. The degree of protection was unaffected by prior infection with parainfluenza virus Type 3, suggesting that such exposure did not enhance exudation of circulating immunoglobulin into the respiratory tract. It was concluded that systemic humoral immunity alone can prevent pasteurellosis.

### INTRODUCTION

Pneumonic pasteurellosis of sheep generally involves *Pasteurella haemolytica* Biotype A, though predisposing factors may also be significant (Gilmour, 1978). Disease indistinguishable from the natural condition has been reproduced experimentally in specific pathogen-free (SPF) lambs using aerosols of *P. haemolytica*, either alone or preceded by parainfluenza virus Type 3 (PI3) (Gilmour et al., 1975; Sharp et al., 1978).

Several vaccines incorporating bacterial cell extracts have been shown to be protective against homologous serotype challenge (Gilmour et al., 1982; Wells et al., 1984), but the A2 serotype was poorly immunogenic in lambs (Gilmour et al., 1983), mice (Evans and Wells, 1979) and rabbits (Donachie et al., 1986a).

The reasons for the lower immunogenicity of the A2 serotype are unknown, but the practical implications are considerable: A2 constituted 55% of biotype A isolates in one survey of pneumonia in British sheep (Fraser et al., 1982).

The mechanisms by which the efficacious vaccines protect sheep are not yet understood. Wells et al. (1979) failed to protect SPF lambs with serum from lambs that had been vaccinated against Serotype A1, which led them to conclude that humoral immunity alone was incapable of protecting against experimental pasteurellosis.

Serum from sheep convalescent from experimental pneumonia involving *P. haemolytica* A2 and *Mycoplasma ovipneumoniae* (Jones et al., 1986) passively protected mice against experimental pasteurella infection (Donachie et al., 1986b), whereas antiserum to inactivated antigens did not. A re-evaluation of the protection afforded by transferred serum to ovine pasteurellosis using serum from both convalescent and vaccinated sheep seemed justified and is described in this paper.

## MATERIALS AND METHODS

### *Experimental animals*

#### *Serum donors*

All animals used were conventionally reared Cheviot lambs from the same flock. Though the flock was virtually free of respiratory disease during more than 9 years of close observation, *P. haemolytica* had been found occasionally in nasal swabs.

While under experiment, the convalescent groups (Cv1 and Cv2) were maintained indoors in isolation. The vaccinated animals (Groups V1 and V2) were maintained outdoors throughout as distinct, separate groups. Each group contained six lambs of 3.5–4 months old (m.o.) at experiment initiation.

In pre-experimental screening, *P. haemolytica* was isolated from nasal swabs from two sheep in Group V1 and titres of antibody to *P. haemolytica* were detected by anti-sodium salicylate extract (SSE) ELISA ( $>1$  in 500; Donachie and Jones, 1982) in two lambs in each of Cv1, V1 and V2, and in three lambs of Cv2.

#### *Serum recipients*

Crossbred SPF lambs, produced and maintained as described previously (Hart et al., 1971), were randomised into groups on the basis of liveweight, with restriction on siblings.

### *Production of immune serum*

#### *Convalescent groups*

Chronic ("atypical") pneumonia was reproduced experimentally at different times in two groups of lambs by minor variations of the general methodology described elsewhere (Jones et al., 1986). Briefly, homogenised lung lesion suspensions made from naturally occurring cases of atypical pneumonia (LH) were injected intratracheally (i.t.) in 8–10-ml volumes on Day 0. Two different suspensions were used. The first (injected into Group Cv1) was treated with 2 mg ml<sup>-1</sup> of ampicillin (Penbritin, Beecham Animal Health) and contained *M. ovipneumoniae* as the only detectable microorganism; present at 10<sup>6</sup> colour-changing units (ccu) per 0.2 ml. The second, which was not treated with antibiotic, was injected into Group Cv2 and contained *M. ovipneumoniae* (10<sup>6</sup> ccu per 0.2 ml), *Mycoplasma arginini* (10<sup>5</sup> ccu per 0.2 ml) and *P. haemolytica* A2 [10<sup>3.9</sup> colony-forming units (cfu) ml<sup>-1</sup>].

Group Cv1 was injected intramuscularly (i.m.) with 5 mg kg<sup>-1</sup> liveweight of ampicillin at the time of LH inoculation. The same lambs were injected again on Days 7, 50, 56 and 93 with 2 ml i.t. and 2 ml intranasally (i.n.) of 5-h broth cultures of *P. haemolytica* A2 diluted appropriately in phosphate-buffered saline, pH 7.2. These inocula contained 10<sup>4.5</sup>, 10<sup>4.5</sup>, 10<sup>6.3</sup> and 10<sup>8.0</sup> cfu ml<sup>-1</sup>, respectively.

Group Cv2 was inoculated in like manner on Days 4 and 40 with inocula containing 10<sup>7.2</sup> and 10<sup>5.6</sup> cfu ml<sup>-1</sup>, respectively, of *P. haemolytica* A2.

#### *Vaccinated groups*

Individual animals were injected subcutaneously three times, on each occasion with 2 ml of vaccine prepared by adsorption of antigen with aluminium hydroxide gel (Alhydrogel, Miles Research Products) and emulsification with Bayol and Arlacel A. Group V1 (contemporary with Group Cv1) was injected with a mixture of heat-killed organisms (HKO, 1 mg ml<sup>-1</sup>) and SSE (2.5 mg ml<sup>-1</sup>) of *P. haemolytica* A2 (Gilmour et al., 1983). Each dose was administered 28 days apart. Group V2 (contemporary with Group Cv2) was injected with a vaccine containing SSE only (2.5 mg ml<sup>-1</sup>) given 21 and 22 days apart.

### *Collection of serum and formation of immune serum pools*

Blood in 200–300-ml volumes was taken on four occasions between Weeks 9 and 14, and the animals were exsanguinated in Weeks 14 or 15. Serum pools were formed from all bleedings of the three or four animals in each group that were serologically negative before the experiment.

### *Production of control serum*

Two batches of control sera were used for Experiment 1. One (CtS1) was made up of sera taken from 3–6-week-old (w.o.) SPF lambs; the other (CtS2) was made up of sera taken over 2 weeks from 20–25 conventionally reared lambs aged 4–6 weeks.

For Experiment 2, a serum pool (CtS3) was formed from four peers of those used to produce CvS2 and VS2. These donors were serologically negative when first screened, but low transient titres to *P. haemolytica* were detected by anti-SSE ELISA in three of them during later bleeds.

### *Preparation of semi-purified immunoglobulin-rich fractions*

For Experiment 1, immunoglobulin-rich fractions (IRF) were prepared from ~3-l volumes of CvS1, VS1 and CtS2 by cold ethanol extraction (Cohn et al., 1946; Oncley et al., 1949). These fractions (CvIg1, VIg1 and CtIg2, respectively) were resuspended in 200 ml distilled water and sterilised by passing through filters of 0.22  $\mu\text{m}$  average pore diameter.

IRF for subsequent experiments were prepared by precipitation with 40% (v/v) saturated ammonium sulphate (Garvey et al., 1977) from 1.8-l volumes of CvS2 and VS2 for Experiment 3 (CvIg2 and VIg2i, respectively), and from 1.9 l of VS2 for Experiment 4 (VIg2ii). The precipitates were resuspended in normal saline and dialysed exhaustively against normal saline until free of  $\text{SO}_4^{2-}$  ions as determined by the addition of 10% barium chloride (Garvey et al., 1977). After filtration through 0.45- $\mu\text{m}$  membranes, the suspensions were concentrated using either polyethylene glycol (Breox 20M PEG, Chemical Services and Distribution, Crewe) or lyophilisation followed by resuspension in a minimum volume of distilled water. The suspensions were further dialysed against normal saline, adjusted with saline to a volume considered appropriate for the experiment and sterilised by filtration. Total volumes produced were 250 ml each of CvIg2 and VIg2i, and 380 ml of VIg2ii.

### *Characterisation of serum pools and IRF*

IgG was estimated turbidimetrically (Seneviratne and Moores, 1980) and IgM nephelometrically by an adaptation of the methods of Buffone et al. (1975) using an IL Multistat III microcentrifugal analyser (Instrumentation Laboratory U.K.). Albumin was determined by the bromocresol-green dye binding method of Doumas et al. (1971).

Antibodies to *P. haemolytica* A2 were assayed by indirect haemagglutination (IHA) (Fraser et al., 1983), ELISA using SSE (Donachie and Jones, 1982) or lipopolysaccharide (LPS) bound to assay plates by the method of Fodor and

Donachie (1988), cytotoxin neutralizing (CN) assay (Sutherland et al., 1989) and bactericidal assay (Sutherland, 1988).

### *Passive protection experiments*

The design of these experiments is shown in Table 1.

#### *Infective inocula: administration and dosage*

PI3 virus produced as described previously (Sharp et al., 1978) was injected i.t. and i.n. in 8- and 2-ml volumes, respectively. Two preparations of virus inoculum were used which contained  $10^{6.25}$  and  $10^{6.17}$  TCID<sub>50</sub> per 0.2 ml.

*P. haemolytica* was administered by aerosol as described elsewhere (Gilmour et al., 1975). Titres of *P. haemolytica* from particles  $\leq 3.3 \mu\text{m}$  in diameter, as indicated by the growth from the bottom stage of a 3-stage Porton impinger (May, 1966), ranged from  $10^{6.98}$  to  $10^{8.43}$  cfu ml<sup>-1</sup>.

Virus, when used, was given 7 days before the pasteurella. Day 0 was the day of first exposure to either agent.

TABLE 1

Design of experiments

Experiment	Lambs at Day 0		Challenge method	Treatment of Group				Dose (ml kg <sup>-1</sup> )	
	Age (days)	Weight (kg)		1	2	3	4	i.p. (S)	i.v. (Ig)
1	67-69	12.5-19.5	PI3+PhA2	CvS1+ CvIg1 (614)*	VS1+ VIg1 (628)	CtS1+CtS2 +CtIg2 (444)		22	1.4
2	25-26	5.7-13.9	PI3+PhA2	CvS2 (542)	CtS3 (624)			20	
3	20-21	5.9-11.2	PI3+PhA2	CvIg2 (451)	VIg2i (664)	VS2 (365)	None	11.4 (S i.v. and i.p.) <sup>2</sup>	3 <sup>1</sup>
4	30-31	5.2-14.1	PhA2 only	VIg2ii (606)	None				4.1

Cv = convalescent; V = post-vaccinal; Ct = control; S = serum; Ig = immunoglobulin-rich fraction; i.p. = intraperitoneal injection given 24 h before challenge with *P. haemolytica*; i.v. = intravenous injection given 3-4 h before *P. haemolytica*; PI3 = parainfluenza 3 virus; PhA2 = *P. haemolytica* A2.

\*Figures in parentheses indicate total IgG administered as mg kg<sup>-1</sup> liveweight.

<sup>1</sup>Groups 1 and 2.

<sup>2</sup>Group 3 was given 11.4 ml serum kg<sup>-1</sup>, 30 ml i.v. and the remainder i.p.



Large volumes (60–430 ml) of serum were administered 24 h before *P. haemolytica* infection by intraperitoneal (i.p.) injection into the right sublumbar fossa using a flutter valve. Small volumes of IRF (18–59 ml) or serum (30 ml, Group 3, Experiment 3) were given by intravenous (i.v.) injection 3–4 h before exposure to aerosol. In Experiment 4, where i.v. doses were particularly large, the animals were pre-sedated with Saffan (Glaxovet). Serum or IRF was warmed to 37°C before injection.

*Clinical, microbiological, histopathological and serological examinations and necropsy*

A combined scoring (CS) system was employed, based on four components, in which the maximum score per lamb was 60.

(i) Clinical score. Animals were examined from Days 1 to 6 inclusive and necropsied on Day 7. One point was allotted for each observation of raised rectal temperature ( $\geq 40.5^\circ\text{C}$ ), dyspnoea or coughing, severe depression, and death or euthanasia on humanitarian grounds. The maximum daily score was 4 and the experiment score 24. Animals which died prematurely were scored 4 for each day remaining in the experiment.

(ii) Pneumonia score. The mean area of consolidated lung tissue on dorsal and ventral surfaces was assessed by computer-aided measurement from lung diagram charts completed at necropsy. This area, expressed as a percentage of the total lung area, was halved to give the pneumonia score (maximum 22). Animals which died rapidly, without development of consolidated lung tissue, were scored maximum if isolations of *P. haemolytica* indicated lung infection and a generalised septicaemia (one or more of heart blood, spleen and liver positive).

(iii) *P. haemolytica* isolation index. Lung tissue samples from eight different sites were combined to give four pools as follows: right apical and cardiac lobes; left apical and cardiac lobes; right diaphragmatic and intermediate lobes; and either side of the left diaphragmatic lobe. These samples were titrated and cultured, and the counts of viable *P. haemolytica* estimated. Representative colonies were identified by methods described elsewhere (Fraser et al., 1983). The mean  $\log_{10}$  titre of the four pools was taken as the isolation index figure (maximum 8).

(iv) Pleurisy. Fibrinous pleuritic adhesions were scored 1 for each lobe affected, the apical and cardiac lobes of each side being regarded as one lobe for this purpose (maximum 4). Gelatinous fibro-cellular pleurisy with excess pleuritic fluid was scored 3 for each side affected (maximum 6).

Surviving lambs were killed at Day 7 by barbiturate overdosage. Lung tissue pools were examined for bacteria and viruses as described previously (Sharp et al., 1978), and representative lung blocks were fixed in 10% formol saline for histopathological examination. Sera were examined for antibodies to *P. haemolytica*.



## RESULTS

*Characteristics of the serum pools and IRF (Table 2)*

IgG:albumin ratios were 0.56–1.20 in whole serum. They were increased 9.9–11.0-fold by cold ethanol extraction and 6.7–8.2-fold by ammonium sulphate precipitation. IgM:albumin ratios were increased 8.5–8.7-fold by cold ethanol extraction and 3.0–4.8-fold by ammonium sulphate precipitation.

All sera and IRF pools from vaccinated or convalescent animals were positive and those from control animals negative by anti-SSE ELISA; IRF demonstrated higher titres than their parent serum pools. All pools tested by anti-LPS ELISA were positive, including CtS3 which had a low titre. The CN assay produced very similar findings, except that VS1 was negative. The bactericidal assay distinguished immune from control serum and IRF, although some bactericidal activity was detected in CtIg2 and none was detected in CvIg2 or VIg2(i). The IHA test distinguished immune from control serum, except in Experiment 1, where three immune products yielded insignificant titres. In

TABLE 2

Characterisation of serum pools and immunoglobulin-rich fractions

Experiment	Group	Inoculum	Content (g l <sup>-1</sup> )			Reciprocal of titre*			Bacteri- cidal assay (%K)	
			IgG	IgM	Albumin	IHA	ELISA			CN
							SSE	LPS		
1	1	CvS1+	26.0	0.7	27.1	16	1172	6500	200	100
		CvIg1	30.3	0.7	3.2	2	5434	3111	800	100
	2	VS1+	26.7	0.7	35.6	0	2597	12091	<100	86
		VIg1	29.1	0.6	3.5	2	9060	10292	100	100
	3	CtS1+2	17.6	0.2	31.7	2	0	NA	<100	0
		+CtIg2	40.5	1.2	4.1	0	0	NA	<100	36
2	1	CvS2	27.1	0.7	30.2	32	1046	3183	200	91
	2	CtS3	31.2	0.7	31.1	2	0	2615	<100	0
3	1	CvIg2	150.2	1.5	21.8	128	2748	NA	1600	0
	2	VIg2(i)	221.3	1.6	22.4	32	62502	24369	1600	0
	3	VS2	32.0	0.4	26.7	8	9060	6697	100	90
4	1	VIg2(ii)	147.9	1.0	18.5	32	41996	8342	400	88

\*Mean of duplicate tests using *P. haemolytica* A2 as antigen. IHA=indirect haemagglutination test; ELISA=enzyme-linked immunosorbent assay; CN=cytotoxin neutralisation assay; SSE=sodium salicylate extract; LPS=lipopolysaccharide; %K=percent killed; NA=not available. Otherwise, abbreviations as in Table 1.

general, the highest titres in the IHA test were produced by convalescent sera and IRF.

### *Experiments 1 and 2*

Virtually no evidence of pneumonia was detected in the 19 animals treated with immune material and only one (from Experiment 2) yielded *P. haemolytica* from the lungs, in low numbers (Table 3). In contrast, three of the six control lambs in Experiment 1 died rapidly and four had consolidated lesions in the lungs.

All six control lambs in Experiment 2 yielded *P. haemolytica* from the lungs ( $P < 0.01$  compared with Group 1), but marked evidence of pneumonia was apparent in only two, of which one died and one had extensive lung consolidation.

### *Experiment 3*

Three lambs died of PI3 virus infection on Days 5 and 6. The remainder were then randomised on pre-experiment weights into three groups of seven and one (Group 4) of eight lambs for challenge with *P. haemolytica*.

No deaths occurred in any of the treated lambs and evidence of disease in them was minor (Table 3). Mean isolation titres were  $< 10^2$  cfu g<sup>-1</sup> in all six of the 21 animals treated with immune serum which yielded *P. haemolytica* from the lungs. In contrast, seven control lambs died, all with evidence of septicaemia. The sole survivor yielded a mean titre of only  $10^{2.4}$  cfu *P. haemolytica* g<sup>-1</sup> of lung tissue, but had consolidation involving 94% of the lung surface area.

### *Experiment 4*

Clinical signs of disease were virtually absent in the group treated with immune Ig, although three lambs showed 5–8% consolidation of the lung surface area; two of these yielded low numbers of *P. haemolytica* (Table 3). All but one control animal died of septicaemia, most on Day 3. *P. haemolytica* was isolated from a shoulder joint, but not the lungs of the sole survivor.

Histopathologically, the majority of animals in Experiments 3 and 4 showed interstitial pneumonia with or without a non-suppurative exudate. A small proportion, represented in every group, showed mild to moderate neutrophil presence with or without lung consolidation. Changes typical of pasteurellosis were found in only one animal of Group 3, Experiment 3. Fibro-cellular pleurisy was observed only in untreated animals, with four positive in both Experiments 3 and 4.

Sera of lambs in Experiments 3 and 4 taken after treatment, immediately before challenge with *P. haemolytica*, were assayed by anti-SSE ELISA (Table

TABLE 3

Clinical and necropsy findings in Experiments 1, 2, 3 and 4

Experiment	Group	N	Source of treatment materials	No. dying or killed in extremis	Mean clinical score (24) <sup>1</sup>	Mean pneumonia score (22) <sup>1</sup>	No. with pleurisy	No. with <i>P. haemolytica</i> in lungs	Mean CS ± SE	%P
1	1	6	Convalescent (S+Ig)	0	0	0	0	0	0	100
	2	6	Vaccinate (S+Ig)	0	1.0	0.2	0	0	1.2 ± 0.4	95
	3	6	Control (S+Ig)	3	9.5	9.5	2	4	24.7 ± 10.1	0
2	1	7	Convalescent (S)	0	0.3	0	0	1**	0.7** ± 0.4	94
	2	6	Control (S)	2	2.7	5.7	1	6	12.7 ± 3.8	0
3	1	7	Convalescent (Ig)	0**	0.9**	0.3**	0*	1**	1.3** ± 0.5	97
	2	7	Vaccinate (Ig)	0**	0.3**	0.3**	0*	3	1.1** ± 0.5	97
	3	7	Vaccinate (S)	0**	0.4**	0.6**	1	2*	1.1** ± 0.6	97
	4	8	None	7	11.8	22	5	8	43.1 ± 3.5	0
4	1	8	Vaccinate (Ig)	0***	0.4***	1.4**	2*	2*	2.5** ± 1.1	95
	2	9	None	8	16.8	19.6	7	8	47.0 ± 5.6	0

CS=combined score (maximum=60). Otherwise, abbreviations as in Table 1.

$$\%P = \text{Protection} = 100 \left( 1 - \frac{\text{test CS}}{\text{control CS}} \right)$$

<sup>1</sup>Figures in parentheses indicate maximum possible score.

\*P<0.05; \*\*P<0.01; \*\*\*P<0.001, all with respect to the untreated control group within the relevant experiment according to the Mann-Whitney two-tailed test.

TABLE 4

ELISA titres to *P. haemolytica* in the sera of lambs in Experiments 3 and 4 following treatment, immediately before challenge with *P. haemolytica*

Experiment	Group	Treatment	Anti-SSE ELISA	
			No. + ve <sup>1</sup> / No. tested	Titre ( $\pm$ SE) <sup>2</sup>
3	1	CvIg2 i.v.	0/7	0
	2	VIg2i i.v.	6/7	3.1 $\pm$ 0.06
	3	VS2 i.p. + i.v.	1/7	2.8
	4	None	0/8	0
4	1	VIg2ii i.v.	5/8	3.1 $\pm$ 0.10
	2	None	0/9	0

<sup>1</sup>Titres  $\geq 1$  in 500.

<sup>2</sup>As log<sub>10</sub>, positive animals only.

4). Most lambs treated with VIg2 were positive, but none injected with CvIg2 and only one given VS2 had detectable antibody.

#### DISCUSSION

These studies have demonstrated that serum or IRF administered i.v. or i.p. can provide virtually total protection against experimental pasteurellosis. Protection was produced by two separate batches of convalescent serum, by post-vaccinal serum produced with two forms of vaccine and by IRF produced from these convalescent and post-vaccinal sera. These findings thus support other work indicating that protection against respiratory disease can be mediated by humoral elements alone (Lam and Switzer, 1971; Masiga et al., 1975), in contrast to the conclusion of Wells et al. (1979) that humoral immunity per se is incapable of preventing pneumonia, specifically ovine pasteurellosis.

Gilmour et al. (1983) found that Serotype A2 SSE/HKO vaccines similar to those used in this study gave only 40–50% protection in SPF lambs, whereas Donachie et al. (1986a) observed that SPF lambs convalescent from experimental infection with *P. haemolytica* A2 were completely resistant to subsequent challenge with the homologous serotype. However, in this study similar levels of passive protection (between 94 and 100%) were afforded by serum both from vaccinated and convalescent animals. This anomaly may have been due to several reasons. (i) Excessive dosages of serum and IRF may have obscured differences in levels of protective antibodies raised by these two protocols. Generally, convalescent serum and IRF pools had higher bactericidal as-

say (%K), CN and IHA titres than did vaccinate serum and IRF pools. (ii) A considerable boost in antibody titre was invariably seen in these studies by anti-SSE ELISA following the third vaccination. Gilmour et al. (1983) used only a double-vaccination schedule. (iii) Prior exposure of donor animals to *P. haemolytica* may have supplemented the smaller range of antibodies stimulated by the SSE vaccine, thereby raising the protective efficacy of post-vaccinal serum to equivalence with the convalescent serum. Pre-screening indicated that donor animals were negative for antibodies to *P. haemolytica* A2 by the anti-SSE ELISA, but some of their peers were positive by this test and two yielded *P. haemolytica* from nasal swabs. In Experiment 2 also, control serum from untreated peers of immune serum donors appeared to offer some protection (Table 3) and antibodies were present at low titre to LPS (Table 2). This indicates that the anti-SSE ELISA, probably the most sensitive test employed, lacked the ability and/or sensitivity to detect low levels of protective antibodies. The same test detected circulating antibodies in only 12 of 29 recipient lambs in Experiments 3 and 4 immediately before challenge.

IRF preparations were as effective as their parent serum pools in these studies. Being no more than semi-purified, the precise nature of their protective components cannot be deduced, although compared with serum their proportional contents of IgG and IgM were increased at the expense of albumin and, presumably, other serum components. Since dosages used were equilibrated by IgG content, this suggests that immunoglobulin was responsible for the protection engendered.

The use of a *P. haemolytica*-only infection in Experiment 4 was designed to ascertain whether prior infection with PI3 virus was responsible, wholly or in part, for the protective effects of injected materials seen previously. PI3 virus can cause epithelial damage and oedema (Rushton et al., 1979; Davies et al., 1981), effects which could have promoted the exudation and accumulation in the lungs of blood constituents, including the injected materials. The 95% protection observed in Group 1, Experiment 4, indicates that viral pre-infection was not essential for efficacy of treatment and that either normal immunoglobulin transudation into the broncho-alveolar tree was sufficient or that protection was provided mainly or exclusively intravascularly.

Death after acute or hyperacute septicaemia occurred in some control animals of all experiments, but the 3-4.5-w.o. lambs of Experiments 3 and 4 had virtually no pulmonary phagocytic cell response, compared with the extensive neutrophil and macrophage exudate present in the lungs of three 9.5-w.o. control lambs of Experiment 1. This difference suggests that phagocytic cell function and responses in the young lamb are deficient and mature only after 5 weeks of age. Naturally occurring field cases of pasteurellosis in lambs <2 months old are generally of septicaemic form (Gilmour, 1978). In the absence of severe respiratory disablement, the cause of death in such cases is presumably endotoxaemia. The ability of immune serum and IRF to protect the immunologically immature lambs of Experiments 3 and 4 suggests that protec-



tion was independent of phagocytes, i.e., non-opsonic. Instead, the injected immunoglobulins may have been bactericidal, prevented bacterial adherence, enhanced clearance by the muco-ciliary blanket and/or neutralized bacterial metabolic function or products, such as endotoxin or cytotoxin (Pennington, 1979; Shewen and Wilkie, 1983), by steric hindrance.

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